MODULATION OF NOCICEPTION IN PAINFUL DIABETIC NEUROPATHY

BY

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Abstract

Diabetes mellitus has become a global pandemic, with almost 350 million individuals world-wide affected by the disease. There are two common forms, characterized by either a lack of insulin production or peripheral insulin resistance. Diabetic peripheral neuropathy is the most common and debilitating complication in both forms, and approximately 30% of individuals with diabetic neuropathy will experience pain that significantly impacts their quality of life. Unfortunately, symptomatic treatment modalities are often ineffective and carry significant risk of systemic adverse effects.

The work contained herein used a rodent model of painful diabetic neuropathy following induction of diabetes with the pancreatic beta cell toxin, streptozocin (STZ). Results show that diabetes reduces the ability of specialized enzymes, termed ectonucleotidases, to hydrolyze extracellular AMP into the analgesic small molecule adenosine. At this same time, diabetic mice displayed significant mechanical allodynia, suggesting that altered adenosine production and decreased activation of the antinociceptive A_1 adenosine receptor (A_1R) may contribute to the development of painful diabetic neuropathy.

In the dorsal horn of the spinal cord, A_1R is highly expressed where peripheral pain sensing neurons terminate, placing it in prime location to modulate nociceptive signaling. Central delivery of A_1R agonists, such as AMP, adenosine and the specific A_1R agonist, N^6-cyclopentyladenosine (CPA), significantly improved mechanical withdrawal thresholds in diabetic mice to levels that were not significantly different from nondiabetic mice. A_1R is a G-

protein coupled receptor whose activation results in initiation of downstream second messenger systems. Inhibition of cAMP production and robust activation of Akt were observed following central delivery of adenosine and CPA, suggesting these pathways contribute to the antiallodynic effects of activation of A_1R.

Central delivery of A_1R agonists is not ideal for translation to the human population. Accordingly, peripheral delivery methods were evaluated to determine the efficacy of this therapeutic intervention in painful diabetic neuropathy. Surprisingly, peripheral delivery of CPA resulted in significant improvement in mechanical allodynia in diabetic mice, confirming that the A_1R-mediated antinociceptive pathway is accessible from the periphery. These observations warrant further investigation into this endogenous antinociceptive pathway for the development of novel therapeutic treatment options for individuals suffering from painful diabetic neuropathy.

Dedication

This thesis is dedicated in loving memory to my grandpa and grandma, Jerome and Alma Katz. Their love for family, life, and service laid the foundation for my passion for these same principles. In their passing they unknowingly directed the course of my future. It is because of the diseases that ultimately claimed their lives that I have dedicated mine to the understanding of neurodegenerative diseases, with the hopes of leaving the world a better place through easing the suffering of those affected by devastating neurological disorders.

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Table of Contents

List of Figures

CHAPTER 1

Introduction

Diabetes Mellitus

Diabetes mellitus has become a global pandemic, with over 347 million people affected world-wide [1]. In the United States more than 25 million people have diabetes (both diagnosed and undiagnosed), and almost 2 million new diagnoses were made in 2010 alone [2]. There is an alarming increase in the rate of pre-diabetes in the adult population with an estimated 35% of the population, or 79 million individuals, having abnormally elevated fasting blood glucose levels and/or hemoglobin A1c levels [2], placing them at increased risk for developing diabetes. Direct and indirect medical costs related to the care and treatment of diabetes exceed $174 billion annually [2].

Diabetes is a complex metabolic disorder that results from impaired insulin secretion or altered insulin sensitivity in peripheral tissues [2-5]. This results in fasting hyperglycemia, and elevated blood glucose levels can affect all tissues of the body. There are two main forms of diabetes mellitus, type 1 diabetes (T1D) and type 2 diabetes (T2D). Both types can result in the same long term sequelae, but they have different underlying pathologies. Type 1 diabetes accounts for 5-10% of all diagnosed cases of diabetes, and is mainly diagnosed in children and young adults [2, 6]. Most individuals world-wide are diagnosed with type 2 diabetes (T2D), which accounts for 90-95% of all diagnosed cases of diabetes [2, 3]. This form of diabetes is primarily diagnosed in adults and is associated with a sedentary lifestyle, obesity, and an unhealthy diet [2, 3]. The prevalence of juvenile T2D in the United States has increased in recent years due to increasing rates of childhood obesity [2, 3, 7].

Type 1 diabetes results from autoimmune destruction of beta cells in the pancreas, the primary insulin-producing cells of the body [6, 8]. Patients with T1D are unable to properly

synthesize insulin and, therefore, are unable to take up glucose resulting in a chronic state of hyperglycemia that leads to muscle and protein catabolism, often resulting in weight loss, polydipsia and polyuria [6]. The primary pathology of T2D is hyperglycemia due to decreased insulin sensitivity in insulin-responsive tissues. Ultimately, this increased insulin resistance results in impaired beta-cell production of insulin at which time patients with T2D become dependent on insulin replacement therapy [2, 3, 5]. Despite the underlying cause, all patients with diabetes are at increased risk for several long-term complications such as vascular disease, including myocardial infarction, stroke and hypertension; kidney disease (diabetic nephropathy); blindness (diabetic retinopathy); and nerve damage (diabetic neuropathy) [2, 3].

Diabetic Neuropathy

Clinical manifestations

Diabetic peripheral neuropathy is the most common and debilitating complication experienced by persons with diabetes [5, 9-11], and it can affect sensory, motor and autonomic peripheral neurons. Shockingly, up to 70% of patients with diabetes suffer complications related to the nervous system [2], affecting patients with both T1D and T2D. The most common manifestation is a length-dependent polyneuropathy (also known as distal symmetric polyneuropathy) that preferentially targets the longest axons of the body first, although other types of neuropathy are also prevalent in diabetes [12]. Due to the length-dependent destruction of nerves, symptoms are often first noticed in the feet where the longest axons of the body terminate, and symptoms can be either focal or diffuse in nature. Symptoms begin in the extremities and then progress proximally in a length-dependent fashion, often referred to as a

stocking-and-glove pattern of sensory loss, with sensory loss predominant over motor symptoms [4, 9]. Patients with diabetic neuropathy experience a variety of symptoms ranging from increased sensations such as tingling, burning, or pins-and-needles sensations (referred to as paresthesias), heightened sensitivity to normally innocuous stimuli (allodynia) or exaggerated responses to normally painful stimuli (hyperalgesia). On the other hand, patients can experience loss of sensation that includes numbness, weakness, and loss of proprioception, placing them at increased risk for falls [5, 9].

Altered sensation as a consequence of diabetic peripheral neuropathy can result in the development of several other physical pathologies. Loss of sensation in the feet can lead to the development of foot ulcers, as patients with diabetic neuropathy are unable to feel the initiation and development of lesions on the feet. If not cared for properly this loss of sensation, in combination with impaired wound healing, can result in the development of severe infections and ulcers, and in extreme cases can lead to amputation. Indeed, diabetic neuropathy is one of the leading causes of non-traumatic lower-limb amputation in the United States [2, 9, 13]. Furthermore, loss of proper nerve function can lead to impaired proprioception, placing patients with diabetes at increased risk for falls [11]. Additionally, sensory loss can lead to the development of Charcot joint, a condition that is due to repetitive trauma and can result in fracture, dislocation, or deformation of the foot [14], which can further contribute to instability and falls. Carpal tunnel syndrome and ulnar nerve entrapment can significantly affect hand function and are more common in patients with peripheral neuropathy [5]. Other conditions secondary to diabetes-induced neuropathy include autonomic neuropathy (primarily affecting the

gastrointestinal tract), radiculoplexopathy (most commonly seen in the lumbosacral region), and other focal neuropathies [5, 9].

Diagnosis

The American Diabetes Association and the American Academy of Neurology both recommend nerve conduction testing as an objective method of detecting peripheral neuropathy in patients with diabetes [12, 15, 16]. This test evaluates nerve conduction velocities in large myelinated fibers by measuring the time it takes for an electrical impulse to travel from the stimulus location to the recording location. Slowing of conduction velocities is indicative of neuropathy. However, diabetic neuropathy affects all fiber types, including large myelinated Aβ-fibers, thinly myelinated Aδ-fibers, and unmyelinated C-fibers (discussed in detail below), and nerve conduction studies are, therefore, limited in that they are able to detect abnormalities only in large fiber types, which are generally affected late in disease progression [17].

To augment the ability to detect changes in the peripheral nervous system, qualitative sensory tests (QSTs) are commonly used in clinical practice as a supplement to nerve conduction studies. Qualitative sensory tests involve application of a stimulus to the foot and its detection by the patient. Mechanical sensitivity, heat and cold thresholds, vibratory sensation, and the presence or absence of ankle and/or lower extremity reflexes are common exams performed by clinicians to diagnose peripheral neuropathy [18-20]. These QSTs are easy to perform and are non-invasive; however, they are inherently variable due to inconsistencies in application and delivery of the exam [15, 21]. Nevertheless, QSTs are a suitable annual testing method for detecting the presence of neuropathy, and multiple positive signs are correlated with high sensitivity and diagnostic accuracy [15, 16].

Patients with small-fiber neuropathy often have normal electrophysiological studies, yet display signs and symptoms of neuropathy [22, 23]. Intraepidermal nerve fiber (IENF) densities have recently been used to identify small-fiber neuropathy and track the progression of diabetic neuropathy. This analysis involves direct visualization of nerve fibers in the skin [22, 24]. A small, 3-mm punch biopsy is taken, typically from the distal portion of the lower leg, and is then processed and stained with antibodies that detect all nerve fibers in the dermis and epidermis. These fibers are then counted to determine the average number of IENFs present in the region from which the skin was taken. If these values are lower than average, or if they decrease after repeated testing, then the patient is diagnosed as having small-fiber neuropathy. This test has relatively high diagnostic accuracy and is more sensitive than QSTs at diagnosing small-fiber neuropathy [22, 24]. It has been suggested that small-fiber neuropathy is one of the earliest signs of impaired glucose tolerance as a high percentage of patients with impaired glucose tolerance exhibit decreased IENF densities [20, 25]. A relatively new approach to identify patients at risk for diabetic neuropathy is corneal confocal microscopy [26]. The cornea is a highly innervated tissue, and contains a very high density of unmyelinated nerve fibers. This noninvasive technique is still in the developmental stages but has shown promise in that it can detect changes in corneal nerve fiber density, length, and branching, and these changes correlate with the severity of neuropathy.

Pathogenesis of Diabetic Neuropathy

Collectively, patients with diabetic neuropathy exhibit a variety of signs and symptoms, owing to damage of the different nerve fibers resulting from chronic hyperglycemia. Indeed, the severity of diabetic neuropathy correlates to the severity and duration of hyperglycemia [20].

Tight glycemic control is currently the only proven disease modifying intervention for delaying progression of diabetic neuropathy [5, 9, 10, 16, 27, 28]. As was eluded to earlier, symmetrical loss of distal axons is a hallmark of diabetic peripheral neuropathy, with both small and large fibers affected [5]. Several histological abnormalities are present, affecting not only the nerves, but also the supporting cells and vasculature supplying the nervous system. Segmental demyelination and remyelination of nerve axons is present, and this can contribute to altered nerve conduction along the axon [4]. Alterations in the vasa nervorum can lead to neuronal ischemia and decreased diffusion of nutrients from the blood supply to the axons due to endothelial basement membrane thickening [4, 5]. Additionally, 'onion bulb' formation is often seen upon morphological examination of peripheral nerves, indicative of chronic and repeated demyelination and remyelination of the nerve [29].

A number of mechanisms underlying the pathogenesis of diabetic neuropathy have been explored, and it is likely a combination of vascular, immunological and metabolic abnormalities that ultimately leads to the development and maintenance of diabetic neuropathy. Excess glucose due to chronic hyperglycemia results in the movement of glucose through several different pathways [10, 20, 30, 31]. Glucose that cannot be broken down through glycolysis can enter the polyol pathway, resulting in increased oxidative stress. Elevated levels of fructose-6-phosphate and glyceraldehyde-3-phosphate, two intermediates of glycolysis, are transported through the hexosamine pathway and protein kinase C pathway, respectively, both resulting in altered gene expression. Glyceraldehyde-3-phosphate, as well as excess fructose from the polyol pathway, can contribute to the formation of advanced glycation end products (AGEs) leading to irreversible protein modifications. Additionally, free radical generation, decreased neurotrophic

support, altered mitochondrial function, and microvascular complications are all thought to contribute to the development of peripheral neuropathy.

Painful Diabetic Neuropathy and Treatment

A significant proportion of patients with diabetes experience pain, with estimates ranging from 10-20% of patients in the general diabetic population, and 40-60% of patients with documented diabetes-induced peripheral neuropathy [9, 17, 32]. This pain can be so severe that it significantly impacts patients' quality of life, affecting their ability to walk, work, sleep, and their overall mood. Furthermore, patients with painful diabetic neuropathy report lower rates of self-management and exercise, and this is correlated with the severity of pain [32]. Additionally, healthcare costs for patients with painful diabetic neuropathy are up to three times higher than matched controls [17]. Individuals suffering from painful diabetic neuropathy often present with lancinating or shooting pain in the feet (paresthesias), allodynia and/or hyperalgesia, and symptoms often worsen at night. Symptoms associated with painful diabetic neuropathy are indicative of small-fiber (Aδ- and C-fiber) involvement.

It is currently unknown why only a subset of patients with diabetic neuropathy develop chronic pain, when all patients presumably exhibit the same underlying mechanisms contributing to the development of peripheral neuropathy. Several treatment options exist; however, few target the underlying pathophysiology implicated in painful diabetic neuropathy. Treatments aimed at alleviating the symptoms associated with painful neuropathy are either insufficient at managing the pain or come with significant risk of side effects. Current treatment algorithms recommend use of oral pharmacologic therapies as first line treatment options for the

management of symptoms; however, none of these medications target the underlying pathologies thought to contribute to the development of painful diabetic neuropathy, highlighting the need to develop pathogenetic treatment options for painful diabetic neuropathy as well as other neuropathic pain conditions [9, 17, 33].

Pathogenetic Treatment

Although current therapeutic options do not address the underlying mechanisms driving painful diabetic neuropathy, several therapies under development are showing significant promise in both cell culture and animal models. One treatment option currently licensed for use in Japan and India is Epalrestat, an aldose reductase inhibitor (ARI). Aldose reductase is an enzyme responsible for converting glucose into sorbitol, the first step in the polyol pathway, and accumulations in sorbitol lead to intracellular osmotic stress which is thought to play a role in nerve damage [30]. Aldose reductase inhibitors have shown some benefit for decreasing pain associated with diabetic neuropathy, however, clinical improvement was not particularly convincing [17, 30].

Use of antioxidants has been evaluated based on the theory that oxidative stress, particularly lipid peroxidation and free radical production, may contribute to the pathology of diabetic neuropathy. This occurs as a consequence of damage to mitochondria as well as inhibition of the vasodilatory effects of nitric oxide, leading to neuronal ischemia and nutrient deprivation. Several countries have approved the use of the antioxidant α-lipoic acid for treatment of painful neuropathy, and two studies (the ALADIN III study, and the SYDNEY 2 Trial) have shown a significant reduction in pain, paresthesias and numbness following intravenous or oral administration of α-lipoic acid, although no improvement in clinical

measurements was seen [34-36]. Another antioxidant, acetyl-L-carnitine, showed a reduction in pain and improvement in vibratory sensation; however, no improvements were seen in nerve conduction velocities [34, 37]. Finally, benfotiamine, a vitamin B1 derivative, showed promising results in a phase III clinical trial, notably improving pain sensation [38]. Collectively, the positive outcomes seen in response to treatment with antioxidants suggests that oxidative stress plays a major role in the development of diabetic neuropathy, and that these compounds could be used as adjunct therapy to minimize pain in patients suffering from painful diabetic neuropathy.

Other pathophysiological treatment options under investigation include the protein kinase C-β (PKC-β) inhibitor, Ruboxistaurin, which, despite promising phase II results, did not have favorable phase III outcomes [39]. C-peptide is part of the pro-form of insulin, and binds to and activates a G-protein couple receptor (GPCR) that leads to activation of phospholipase C (PLC), which in turn leads to increased intracellular calcium levels and activation of endothelial cell nitric oxide synthase (eNOS) and PKC. Activation of PKC, in combination with dacylglycerol (DAG) synthesis, promotes activation of Na^+/K^+ ATPase which improves vascular functioning and nerve conduction velocities. As such, C-peptide replacement therapy has received attention for its potential to improve nerve fiber function (both C-fibers and myelinated fibers) in patients with diabetes [17, 40]. C-peptide, therefore, is ultimately thought to improve nerve conduction in the small-fibers affected by painful diabetic neuropathy.

Symptomatic Treatment

The treatment of painful diabetic neuropathy is centered around the use of pharmacological agents, such as tricyclic antidepressants (TCAs), selective serotonin and norepinephrine reuptake inhibitors (SNRIs), anticonvulsants, and opioids. Currently, there are

only two agents approved by the FDA for the treatment of painful diabetic neuropathy-duloxetine (SNRI) and pregabalin (anticonvulsant), while others are primarily used off-label [17]. Antidepressants have long been used in the treatment of neuropathic pain, and both TCAs and SNRIs are considered first-line treatment options for mitigating pain [33]. Use of TCAs has waned recently in favor of newer drugs that have fewer side effect profiles, such as SNRIs [20, 27, 35]. Some of the first pharmaceuticals developed for the treatment of mood disorders were the TCA's, and this class of antidepressants are some of the best studied in terms of their efficacy for treating neuropathic pain. This class of drugs works by inhibiting the re-uptake of serotonin and norepinephrine, effectively increasing the concentration of these neurotransmitters in the nerve synapse. Amitryptiline, nortriptyline, imipramine and desipramine are the most commonly used TCA's for treating painful diabetic neuropathy. Although efficacious, these drugs come with significant risk of side effects such as weight gain, orthostatic hypotension, cardiac arrhythmias, dry mouth, constipation and somnolence among others. However, these drugs are relatively inexpensive and side effects can be minimized through step-wise increases in effective doses [9, 34, 41, 42]. More recently, the selective serotonin and norepinephrine reuptake inhibitors (SNRIs), such as duloxetine and venlafaxine, have shown beneficial outcomes in reducing perceived pain in patients with painful neuropathy. The major side effects of SNRIs are nausea, drowsiness, constipation, dry mouth and a decreased appetite [34, 41].

Gabapentin and pregabalin are primarily used as anticonvulsants. These medications are calcium channel modulators and work by binding to the $\alpha_2\delta$-subunit of voltage-gated calcium channels, thereby preventing neurotransmitter release. Structurally, gabapentin resembles the neurotransmitter γ-aminobutyric acid (GABA) which is the main inhibitory neurotransmitter in

the central nervous system. Gabapentin has been shown to be effective at reducing neuropathic pain in patients with diabetes compared to placebo, as well as reporting improvements in sleep. However, extremely high doses of gabapentin (up to 3600 mg per day) were needed to achieve efficacy [9, 33, 43]. Pregabalin is a stronger $\alpha_2\delta$-subunit agonist and, therefore, requires much lower doses (300-600 mg per day) to achieve maximum benefit. Pregabalin was effective at reducing mean pain scores in patients with painful diabetic neuropathy; however, there was no improvement in nerve conduction studies [44]. Side effects of both include sedation, dizziness, peripheral edema, headache and weight gain. Both gabapentin and pregabalin are considered first-line treatment options for painful diabetic neuropathy, and pregabalin is one of two drugs approved by the FDA specifically for the treatment of painful diabetic neuropathy [9, 33, 41].

Opioids are commonly used for alleviating pain that can result from a variety of situations. In the case of painful diabetic neuropathy, tramadol, a weak μ-opioid receptor agonist, and controlled release oxycodone, a strong μ-opioid receptor agonist, have been shown to be successful in reducing pain. Caution is advised when prescribing opioids due to their high side effect profile which includes sedation and respiratory dysfunction, drug dependency risk, abuse and misuse potential, changes to the immune system after prolonged use, and decreasing efficacy over time. It is recommended that opioids be reserved for patients who are refractory to first line treatment, or who are suffering from acute exacerbations of neuropathic pain. Combination therapy using gabapentin and morphine showed synergistic effects using lower doses of each medication, and may provide an alternative line of treatment [33, 34, 41, 42].

Several other drugs within each of the aforementioned treatment categories have been evaluated but show less efficacy or inconsistent results to be considered mainstays for the

treatment of painful diabetic neuropathy. Additionally, most pharmacological agents carry risk of systemic adverse events. As such, topical delivery of therapeutic agents has been suggested as a potential treatment modality to alleviate symptoms of painful diabetic neuropathy [45]. Repeated application of 0.05-0.075% capsaicin cream, which effectively depletes the pain-inducing neuropeptide substance P, along with other pro-nociceptive molecules, from cutaneous peripheral nerve endings has mixed outcomes. Recently, use of a single high-dose (8%) capsaicin patch has shown promising results in post-herpetic neuralgia (PHN) and human immunodeficiency virus (HIV)-induced neuropathy [33, 34, 41, 46, 47]. Topical lidocaine decreases the excitability of cutaneous Aδ- and C-fibers, and has been effective at reducing pain associated with PHN and painful diabetic neuropathy. In a 4-week head-to-head study, 5% topical lidocaine plaster was shown to produce results similar to pregabalin at reducing pain and improving quality of life in patients with painful diabetic neuropathy [48].

Despite the numerous aforementioned treatment options for painful diabetic neuropathy, these methods are insufficient for the-long term management of pain symptoms. This highlights the need for the development of new approaches that treat the underlying pathophysiology of painful diabetic neuropathy, rather than generalized symptomatic treatment. Development of novel treatment modalities that activate endogenous antinociceptive pathways, such as that proposed in this dissertation, would provide an efficacious alternative for the treatment of painful diabetic neuropathy.

Nociception: From the Periphery to the Central Nervous System

Pain Sensation

Pain is a complex sensation that involves many types of neurons, receptors and neurotransmitters that are responsible for translating sensory information from the periphery into electrical impulses that are processed by the central nervous system. The International Association for the Study of Pain (IASP) defines pain as, “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.” Pain is the body’s natural warning system and is usually protective in nature. However, when pain is no longer beneficial, the transition from ‘good’ pain to ‘bad’ pain can become particularly disabling and destructive to a person’s quality of life, as is the case with painful diabetic neuropathy. This multifaceted change likely involves several mechanisms operating at multiple levels of nociceptive processing.

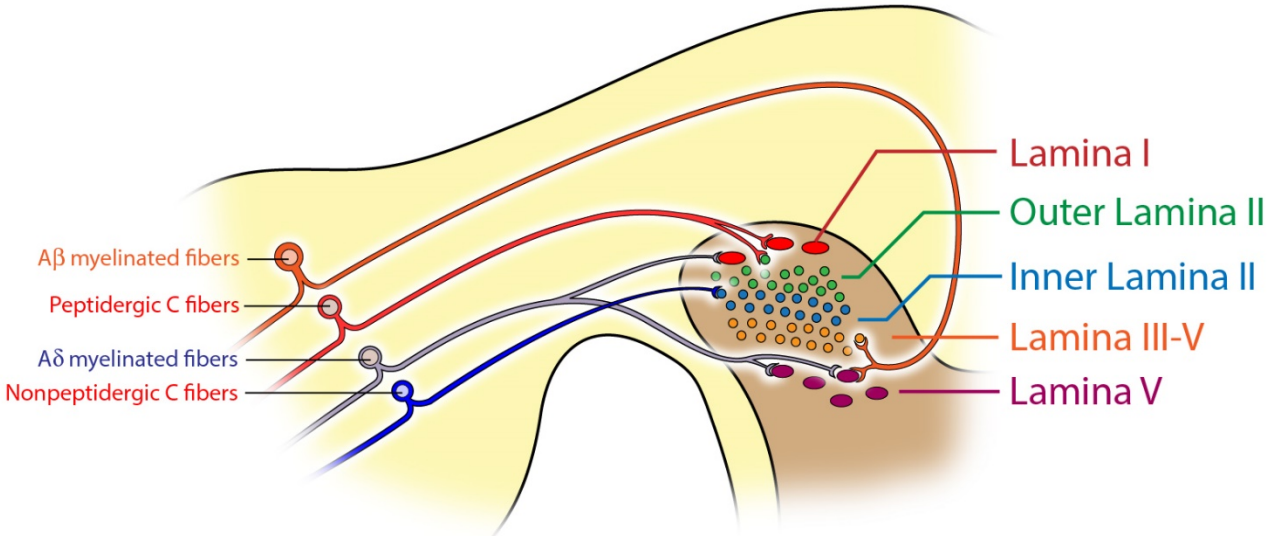
Acute pain is beneficial in that it keeps one from causing serious injury to themselves, or from continuously re-injuring areas that are already damaged. Evidence of the benefit of pain is seen in individuals who cannot sense when they are in danger and consequently injure themselves frequently. Tissue damage leads to the production and release of a host of inflammatory and algesic mediators, such as substance P, prostaglandins, leukotrienes and histamine, resulting in heightened sensitivity of the tissue or area surrounding the wound. Importantly, acute pain usually subsides once the wound has healed. Pathological pain, on the other hand, is pain that has persisted beyond its beneficial stage. Pathological pain is typically chronic in nature (lasting > 3 months), and can persist in the absence of any identifiable trauma or lesion [49, 50].

The IASP defines neuropathic pain as “pain caused by a lesion or disease of the somatosensory nervous system.” Painful diabetic neuropathy is a type of neuropathic pain that involves the peripheral nervous system, and is a type of chronic pathological pain. There are several sites along the neuroaxis where alterations in nociceptive signaling can lead to neuropathic pain, including damage or sensitization at peripheral, spinal or supraspinal sites. Peripherally, nerve injury can lead to neuroma formation which can generate spontaneous activity or elicit sensitization to stimuli. Intact peripheral nerves may also emit spontaneous activity, often due to increased expression or sensitization of the channels they express. Channel sensitization can occur through a decrease in the threshold for activation, or by larger responses to stimulus input. Central sensitization occurs within the spinal cord, primarily in the dorsal horn where peripheral pain sensing neurons terminate, and can arise from inflammation, altered release of neurotransmitters (both excitatory and inhibitory), and altered input to spinal interneurons (ex: disinhibition of inhibitory interneurons) [51].

Pain Transmission

Pain is conveyed from the periphery by specialized nerves termed nociceptors. These nerves contain channels and receptors that detect and respond to specific types of noxious stimuli, primarily thermal, mechanical, and/or chemical stimuli. Nociceptors require a stimulus of sufficient intensity for activation, and this minimum threshold for activation can change under pathological conditions. The majority of nociceptors are categorized as thinly myelinated Aδ-fibers, or unmyelinated c-fibers. The cell bodies of these neurons are located in structures called dorsal root ganglia (DRG) that are located next to the spinal column (Fig. 1). These neurons have

Figure 1. **Fiber types involved in conveying sensory information from the periphery to the central nervous system.** The cell bodies of peripheral sensory nerves are located adjacent to the spinal column in structures called dorsal root ganglia. Several fiber types are responsible for conveying sensory information from the periphery to the central nervous system. Aβ-fibers are the largest fiber type and are wrapped in a thick myelin sheath, which allows for extremely rapid conduction of action potentials along the length of the axon. These neurons are primarily responsible for non-noxious stimuli such as light touch, pressure, and proprioception. Aδ-fibers are thinly myelinated fibers that, together with the unmyelinated C-fibers, are primarily responsible for conveying noxious stimuli from the periphery. Some Aδ- and C-fibers are polymodal nociceptors and can convey noxious thermal, mechanical and chemical stimuli. Each of these different fibers terminates in a characteristic lamina in the dorsal horn of the spinal cord. Aβ-fibers terminate in deep in the dorsal horn, in laminae III, IV and V. Aδ-fibers terminate in laminae I and V, whereas C-fibers terminate in lamina I and outer lamina II (peptidergic), and the inner part of lamina II (nonpeptidergic). This figure was modified from Basbaum et al., 2009 [49].


Aβ myelinated fibers
Peptidergic C fibers
Aδ myelinated fibers
Nonpeptidergic C fibers
Lamina I
Outer Lamina II
Inner Lamina II
Lamina III-V
Lamina V

a single axon that splits into two branches: the peripheral branch, which innervates target tissues in the periphery (ex. skin, muscle), and the central branch, which enters the central nervous system where it synapses with second order neurons in the dorsal horn of the spinal cord. In the mouse, peripheral branches of neurons emanating from lumbar DRG's terminate in the hind paw, and the associated central branches terminate in the medial portion of the dorsal horn [49, 50].

Fiber types involved in pain transmission

There are three main fiber types involved in the transduction of sensory information from the periphery into electrical signals that the body can process. Aβ-fibers are the largest fibers and are surrounded by a thick myelin sheath that allows them to rapidly conduct information. These fibers are primarily responsible for conveying non-noxious stimuli, such as touch and muscle movement. Although these fibers are not directly involved in pain transmission, there is evidence to suggest that following nerve damage these fibers can undergo a phenotypic switch where they begin to express substance P, a known pro-inflammatory molecule involved in pain transmission and sensation [52]. Activation of these fibers leads to substance P release, contributing to transmission of nociception in the periphery [53]. Additionally, under pathological conditions there is evidence to suggest that Aβ-fibers can sprout into the dorsal horn, placing them in close proximity to peripheral pain sensing neurons [52]. As such, Aβ-fibers can be activated following activation of nociceptors, and thus can contribute to pain transmission.

Aδ-fibers are small-diameter, thinly myelinated fibers that are involved in conveying pain sensation from the periphery. These fibers can be further subdivided into either type I or type II fibers. Type I Aδ-fibers have a high heat threshold, but low mechanical and chemical thresholds. Following tissue injury, type I Aδ-fibers have been shown to sensitize, lowering their heat and

mechanical thresholds. Type II Aδ-fibers have a high mechanical and chemical threshold, but have a relatively low heat threshold. Because of these different thresholds within the Aδ-fiber population, it is believed that these fiber types convey different information when responding to a stimulus: type I fibers are presumed to convey acute responses to intense mechanical and chemical stimuli, whereas type II fibers are thought to convey acute thermal information [49].

C-fibers are small-diameter, unmyelinated neurons also involved in pain sensation and transmission. Some C-fibers are polymodal in that they can detect both thermal and mechanical stimuli. C-fibers can be further subdivided into either peptidergic or nonpeptidergic fibers. Peptidergic fibers release the pro-nociceptive neuropeptides substance P and calcitonin gene-related peptide (CGRP), as well as express the TrkA receptor that responds to neurotrophic growth factor (NGF). Nonpeptidergic neurons express the receptor c-Ret, which responds to glial-derived neurotrophic factor (GDNF), neurturin and aretemin. Nonpeptidergic neurons also express the markers isolectin B4 (IB4), the GPCR *Mrgprd*, and the $P2X_3$ receptor. [49, 50, 54]

Each of these fiber types (Aβ-, Aδ- and C-fibers) have central projections that terminate in the dorsal horn of the spinal cord. The dorsal horn is divided into several different laminae based on where certain fiber types terminate (see Fig. 1). The outermost region is often referred to as the substantia gelatinosa, and this is where most peripheral nociceptors terminate. Aδ-fibers primarily project to lamina I as well as deeper into lamina V, and C-fibers project to laminae I and II. C-fiber projection patterns can be further subdivided, with peptidergic neurons predominantly localized to lamina I and the outer portions of lamina II, and nonpeptidergic fibers found in the middle regions of lamina II. Aβ-fibers terminate in the deeper layers of the dorsal spinal cord, projecting to laminae III, IV and V. [49, 50]

Pain and heat information is transmitted up the spinal cord to higher processing centers through the spinothalamic tract [55]. Information from Aδ- and C-fibers is passed to second order neurons via spinal interneurons. These second order neurons then cross the midline of the spinal cord and ascend through the spinothalamic tract where they synapse in the ventroposterior lateral nucleus of the thalamus. Tertiary neurons project out to the cortex where information is processed and integrated, and a reaction occurs in a protective maneuver to prevent further injury or harm from occurring.

Channels involved in pain transmission

Nociceptors contain many channels that are responsible for detecting and transmitting noxious stimuli, and most have a high threshold for activation thus allowing them to detect harmful stimuli. Recent progress has identified specific channels that transmit heat, cold, mechanical and chemical stimuli, but precise assignment of channels to the detection of individual modalities remains complicated. Studies using cell culture and transgenic mice have aided in this discovery, albeit with mixed results. Additionally, there are a variety of channels involved in modulating nerve conduction and regulating neurotransmitter release that also contribute to the detection of environmental stimuli. Furthermore, environmental factors and inflammatory mediators can modulate sensory neuron signaling, adding to the complexity of transmitting nociceptive signals.

Thermosensation

Heat transduction: Sensory neurons are capable of distinguishing between general warmth and noxious heat, which has been reported as 43°C, with temperatures above this threshold perceived as potentially damaging to tissues. Capsaicin, the chemical responsible for

the burning sensation felt in response to eating a hot chili, signals through a member of the transient receptor potential (TRP) ion channel family of the vanilloid 1 subtype (TRPV1). TRPV1 is a non-selective ligand-gated ion channel primarily expressed on small-diameter neurons in the DRG that is highly permeable to calcium cations [56]. TRPV1 null mice display an impaired ability to detect noxious heat [57], linking TRPV1 activity to thermosensation. Subsequent studies have provided further evidence that TRPV1 is involved in mediating inflammation-induced thermosensitivity [58]. Other members of the TRP family have also been suggested to play a role in heat sensation, including TRPV2 (threshold for activation ~52°C), TRPV3 and TRPV4 (thresholds between 25°C and 35°C). [49]

Cold transduction: Another member of the melastatin family of TRP channels, TRPM8, is responsive to cold temperatures and methanol, a natural cooling agent, with a threshold of activation around 25°C [59]. TRPM8 is also a ligand-gated cation channel primarily expressed on a subset of nociceptive neurons, distinct from neurons that express TRPV1. TRPM8 null mice display altered sensitivity to cold over a range of temperature from 30°C to 10°C, but display normal responses to noxious heat [60, 61]. Detection of intense, noxious cold (<15°C) is proposed to occur through a member of the ankyrin family of TRP channels, TRPA1, but this is still controversial based on the behavioral responses of knockout mice [62, 63]. Additionally, mice lacking the sodium channel $Na_v1.8$ show altered response to cold over a range of temperatures [49, 64].

Mechanosensation

Somatosensory detection of mechanical stimuli ranges from light brushing to damaging noxious stimuli. Aβ-fibers, which have a low threshold of activation for mechanosensation,

detect light touch/pressure and vibration through innervations with Pacinian corpuscles, Merkel cells, and hair follicles present in the skin. In contrast, Aδ- and C-fibers, which terminate in the dermis and epidermis, respectively, have a high threshold for detecting mechanical stimuli, and thus serve to detect noxious mechanical stimuli. However, identification of specific channels responsible for transducing mechanical sensation remains largely undetermined. Several candidate receptors have been identified, including members from the TRP family (TRPV4, TRPA1) as well as certain acid sensing ion channels (ASICs; ASIC1, ASIC2, ASIC3). TRPV4 is expressed on small- and large-diameter cells in the DRG, and has been shown to co-localize with a variety of cell types in the skin. Interestingly, TRPV4 is not expressed in the spinal cord, suggesting a peripheral mechanism of action for this putative mechanotransducer [65]. Use of animal models to discern the mechanotransduction properties of channels has provided mixed results, as detection of mechanosensation at a cellular level remains difficult. Theories pertaining to the gating of channels involved in transducing mechanical stimuli have been proposed, including stretch-sensitive channels that open in response to deformations in the membrane bilayer, direct gating resulting from the tethering of mechanosensitive channels to the cellular cytoskeleton or extracellular matrix, or indirect gating through activation of and modulation by signaling intermediates [66].

Chemical Sensation

Chemical sensation involves the detection of irritants, such as endogenous factors that are released following injury and environmental chemicals that activate receptors on nociceptive neurons. Several members of the ‘inflammatory soup’ contribute to chemical sensation, such as protons, ATP, histamine, prostaglandins, bradykinin, substance P and adenosine. Many members

of the TRP family of ion channels detect a variety of chemical irritants, and TRPA1 knockout mice show decreased sensitivity to some chemical irritants. [49]

Receptors involved in nociception can be broadly categorized into either metabotropic or ionotropic receptors. Metabotropic receptors involve second messenger signaling cascades, and include the class of receptors called G-protein coupled receptors (GPCR's). GPCR's characteristically have seven transmembrane domains and an associated heterotrimeric G-protein comprised of three separate subunits: an α-subunit, a β-subunit, and a γ-subunit. Binding of a ligand to the receptor causes a conformational change, which in turn activates the G-protein causing it to dissociate from the receptor. Once activated, the α-subunit dissociates from the βγ-subunit, and both can then go on to activate downstream second messengers that propagate the initial signal. Ionotropic receptors primarily form pores through a cell membrane that, upon ligand binding, can open to allow the movement of molecules from one side of the cell to the other, typically following a concentration gradient.

Purinergic Signaling and Nociception

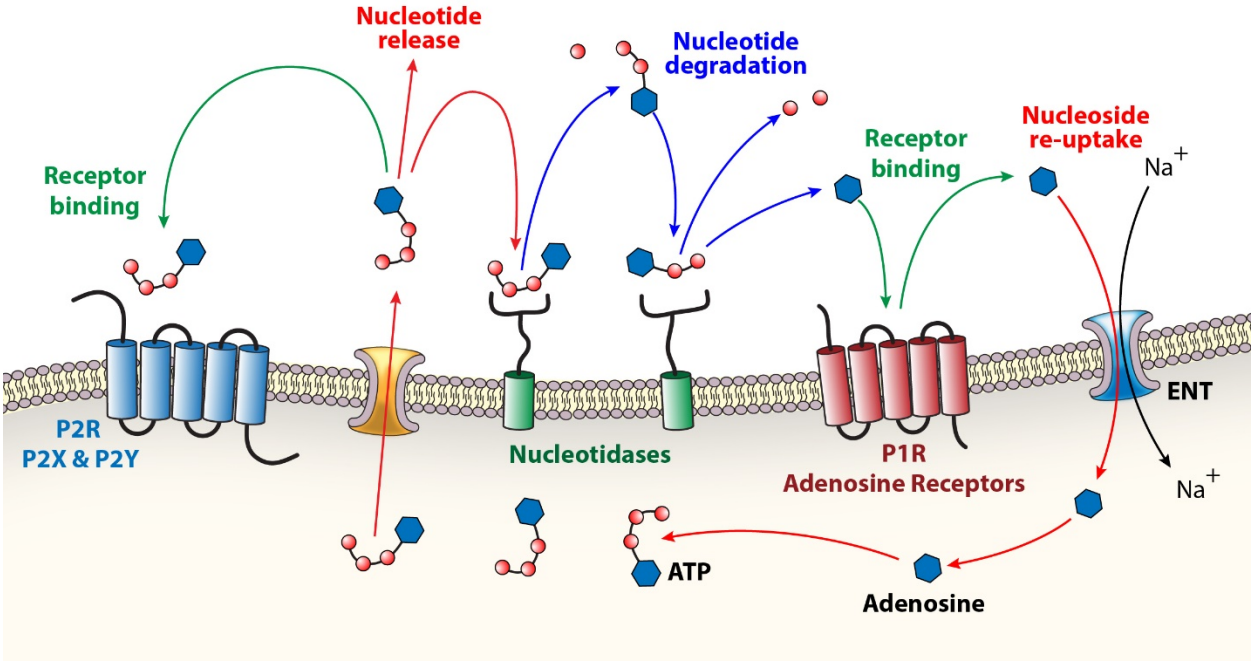
Purinergic signaling, by definition, involves signaling mediated by purine nucleotides and nucleosides, such as adenosine and adenosine triphosphate (ATP) [67]. The concept of purinergic signaling was first proposed in the 1970's based on the observation that ATP could act as a transmitter in both the central and peripheral nervous systems. It is now recognized that ATP can act as both a neurotransmitter and as a source of energy for cellular processes. Identification of adenosine (P1) and ATP (P2) receptors has led to an explosion of interest in purinergic signaling, particularly in the setting of disease, and the potential of these signaling

pathways for modulating disease pathology. Extracellular ATP is rapidly degraded by specialized enzymes termed ectonucleotidases (see discussion below) that terminate ATP-induced pro-nociceptive signaling, and also generate intermediate hydrolysis products, such as adenosine diphosphate (ADP) and adenosine monophosphate (AMP), which in turn can activate additional P2 receptors (Fig. 2).

ATP-mediated Signaling

ATP is stored intracellularly at concentrations around 2-5 mM, and concentrations in synaptic vesicles can be as high as 100 mM. ATP release in neurons is presumed to occur through the fusion of synaptic vesicles at nerve terminals (alone or in combination with other neurotransmitters such as acetylcholine, CGRP and substance P, dopamine, GABA or glutamate, and release can occur in response to exercise, mechanical deformation, osmotic swelling, ischemia or from damaged and dying cells. Thus, ATP signaling has been shown to be involved in a variety of physiological processes such as synaptic transmission, platelet aggregation, immune modulation, cell proliferation, migration and differentiation, and pain signaling [67]. Purinergic signaling has also been implicated in a variety of pathological conditions, especially in the central nervous system (CNS). Altered purinergic signaling is seen following CNS trauma and cerebral ischemia; in neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, multiple sclerosis, and diabetes; in seizure disorders and migraine; in depression and anxiety, including the adenosine-mediated antidepressive effects of some medications (ex: TCAs, SNRIs); and, relevant to this body of work, in neuropathic pain. [68]

Figure 2. Overview of purinergic signaling. Adenosine and ATP are synthesized intracellularly, and then released into the extracellular space following tissue damage or injury, or through nucleotide transporters present in the cell membrane. Once released, nucleotides can activate the P2 family of receptors: the ionotropic P2X and metabotropic P2Y receptors. Alternatively, nucleotides can by hydrolyzed by ectonucleotidases, specialized enzymes that have their catalytic domain oriented towards the outside of the cell. In the case of ATP, hydrolysis by ectonucleotidases results in the production of adenosine, which can then go on to activate the P1 family of receptors, also known as the adenosine receptor family. This figure was modified from Roman and Fitz, 1999 [69].


Nucleotide release
Nucleotide degradation
Receptor binding
Receptor binding
Nucleoside re-uptake
Na+
ENT
P2R
P2X & P2Y
Nucleotidases
P1R
Adenosine Receptors
Na+
ATP
Adenosine

ATP acts on P2 receptors, which are further subdivided into ionotropic P2X or metabotropic P2Y receptors. There are currently seven recognized P2X receptors ($P2X_{1-7}$), and all are ligand-gated ion channels that are activated by ATP. P2X channels are highly permeable to Ca^{2+}, and to a lesser extent K^+ and Na^+. P2X subunits have two transmembrane domains, with the NH_2- and COOH-termini of these receptors located intracellularly, and P2X channels are presumed to form through the assembly of three or four subunits [70]. All P2X receptor subtypes have been detected in sensory neurons, and many studies have documented their role in pain transmission. In the DRG, $P2X_3$ shows the highest expression, and is primarily expressed on $IB4^+$ nonpeptidergic neurons. $P2X_4$ has been linked to mechanical allodynia following nerve injury, and expression of $P2X_4$ is markedly up-regulated in activated microglia in the dorsal horn of L5 nerve injured rats [71]. Furthermore, blockade of this receptor using pharmacological antagonists reversed the nerve injury-induced mechanical allodynia. [71]

There are eight recognized P2Y receptors ($P2Y_1$, $P2Y_2$, $PY2_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$). All are GPCRs and have their NH_2-terminus domain located on the extracellular side of the membrane, while the COOH-terminus is located intracellularly. P2Y receptors can be subdivided based on the G-protein associated with each receptor. $P2Y_1$, $P2Y_2$, $PY2_4$, and $P2Y_6$ are associated with $G_{q/11}$; $P2Y_{11}$, can couple with both $G_{q/11}$ and G_s; $P2Y_{12}$ and $P2Y_{13}$ couple to G_i; and $P2Y_{14}$ is coupled to $G_{i/o}$. Alternatively, the P2Y receptors can be classified based on the ligand for activation: $P2Y_1$ preferentially responds to ADP over ATP; $P2Y_2$ and $P2Y_4$ receptors are equally activated by ATP and UTP; $P2Y_6$ receptors are selectively activated by UDP; $P2Y_{12}$ and $P2Y_{13}$ are preferentially activated by ADP; and $P2Y_{14}$ is selectively activated by UDP-glucose. [72]. P2Y receptors are expressed on small-, medium- and

large-diameter neurons in the DRG. Recently, $P2Y_{12}$ was suggested to play a role in pain signaling. $P2Y_{12}$ expression was shown to be upregulated in activated microglia following nerve injury in rats [73], and studies using antagonists to $P2Y_{12}$ showed diminished development of neuropathic pain following spinal nerve injury [74] or spared nerve injury models of neuropathic pain [73] in mice and rats, respectively.

Integrating information related to altered purinergic signaling and its contribution to the development of neuropathic pain has made great strides in recent years. Following nerve injury, ATP and other nucleotides are released from damaged and dying cells leading to activation of sensory neurons that contain ATP-sensitive receptors, such as those found on Aδ- and C-fibers, and this increases nociception, nociceptor sensitization, and neuropathic pain. In diabetic neuropathy, changes in neuronal support in the setting of chronic hyperglycemia and inflammation underlie mechanisms that contribute to the 'dying-back' neurodegenerative phenotype. Upregulation of pro-nociceptive receptors, such as $P2X_3$, $P2X_4$, $P2X_7$ and $P2Y_{12}$ have been shown to be involved in neuropathic pain models, and could contribute to the development of painful diabetic neuropathy, especially given the likelihood of increased ATP present to activate these receptors.

Adenosine Signaling and Receptor Activation

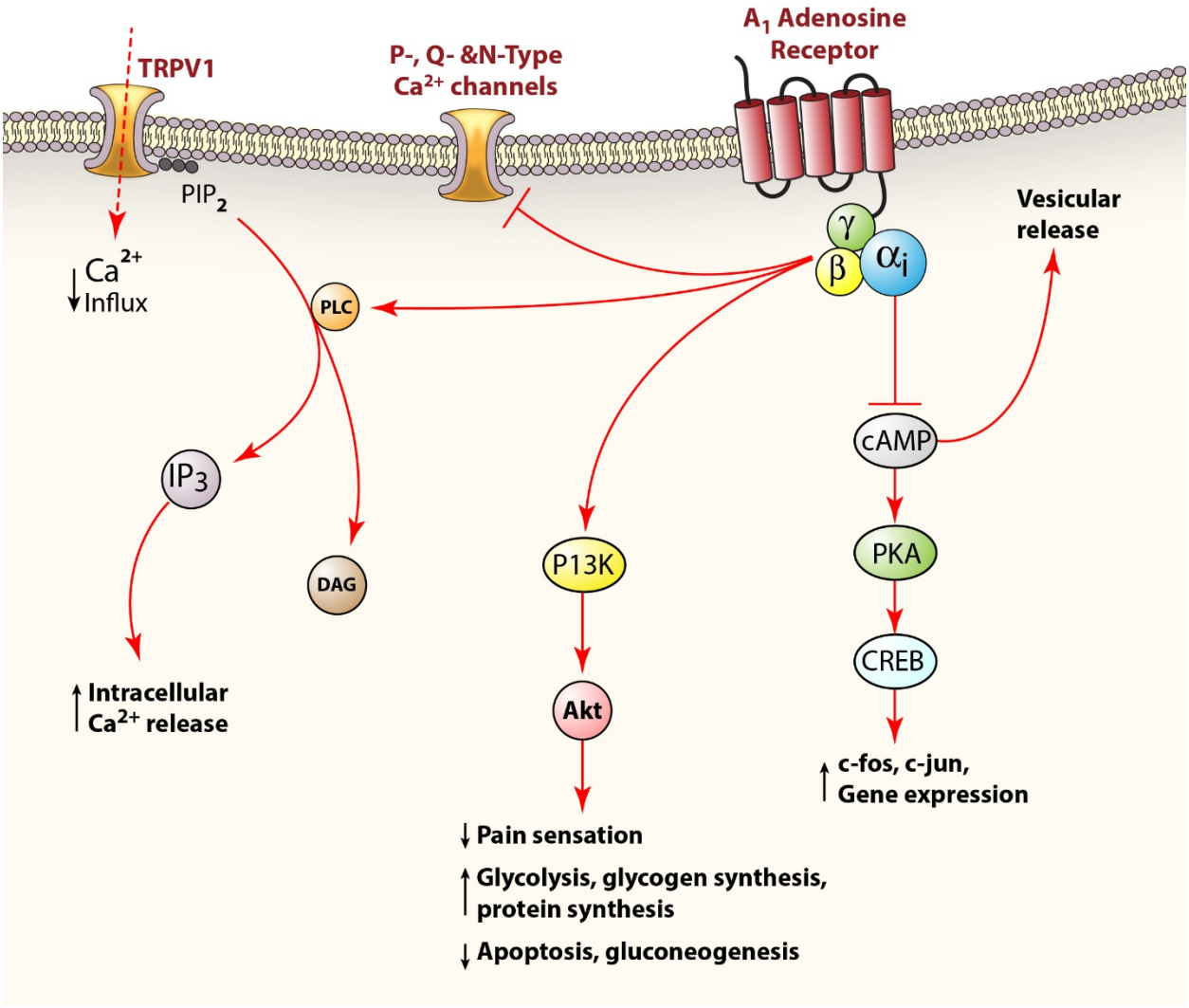
Extracellular adenosine comes from two main sources: release of intracellular stores through membrane-bound channels called equilibrative nucleotide transporters (ENTs), or through extracellular hydrolysis of adenine nucleotides by ectonucleotidases. Unlike ATP, adenosine is not stored in or released from synaptic vesicles. Basal physiological levels of extracellular adenosine are estimated to range from 30-200 nM [75]. Once released, adenosine

can modulate neuronal signaling presynaptically through inhibition or facilitation of transmitter release, or postsynaptically by causing hyperpolarization or depolarization of postsynaptic neurons, owing to the observation that adenosine receptors are found on both pre- and post-synaptic nerve terminals. Additionally, adenosine levels can be modulated through inhibition of adenosine kinase (preventing the conversion of adenosine into AMP) or adenosine deaminase (preventing the deamination of adenosine into inosine), or through inhibition of ENTs, effectively increasing the level of extracellular adenosine allowing for increased activation of adenosine receptors. [76, 77]

Adenosine acts on the P1 class of receptors (adenosine receptors), with downstream effects dependent on the specific receptor subtype activated (Fig. 3). There are currently four recognized adenosine receptors (A_1, A_{2A}, A_{2B} and A_3), all of which are GPCR's. The adenosine A_1 receptor (A_1R) and the adenosine A_3 receptor (A_3R) are $G_{i/o}$-coupled receptors and activation results in inhibition of adenylyl cyclase with subsequent decreases in 3',5'-cyclic adenosine monophosphate (cAMP) production, whereas the adenosine A_{2A} receptor ($A_{2A}R$) and the adenosine A_{2B} receptor ($A_{2B}R$) are G_s-coupled receptors, which stimulate adenylyl cyclase activity and increase cAMP production. Most of the literature thus far has focused on the seemingly antagonistic actions of A_1R and $A_{2A}R$, with much less known about $A_{2B}R$ and A_3R.

The A_1R is highly expressed in the central nervous system, including the cortex, cerebellum and hippocampus, as well as in the dorsal horn of the spinal cord. In the DRG, A_1R is located on small- and medium-diameter neurons, and is found both pre- and post-synaptically as well as on interneurons. Adenosine can act in a manner similar to that of the major inhibitory neurotransmitter, GABA, in that activation of A_1R leads to suppression of excitatory signaling

Figure 3. Signaling downstream of A_1R. A_1R is a GPCR, and activation of A_1R results in inhibition of adenylyl cyclase, with subsequent inhibition of cAMP production, via activation of the $G\alpha_i$ subunit of the associated heterotrimeric g-protein. Activation of the $\beta\gamma$-subunit of the heterotrimeric g-protein results in inhibition of P-, Q- and N-type Ca^{2+} channels, as well as activation of PLC. PLC activation results in cleavage of PIP_2 into DAG and IP_3. PIP_2 has been shown to modulate TRPV1 signaling, and cleavage of PIP_2 results in decreased calcium flux through TRPV1. Abbreviations: GPCR—G-protein coupled receptors; cAMP 3',5'-cyclic adenosine monophosphate; PLC—phospholipase C; PIP_2—phosphatidylinositol 4,5-bisphosphate; DAG—diacylglycerol; IP_3—inositol 1,4,5-triphosphate; TRPV1—transient receptor potential vanilloid 1 type. This figure was modified from Jacobson et al., 2009 [78].


TRPV1
P-, Q- &N-Type
Ca2+ channels
A1 Adenosine
Receptor
PIP2
Ca2+
Influx
PLC
γ
β
αi
Vesicular
release
IP3
DAG
cAMP
P13K
PKA
CREB
Intracellular
Ca2+ release
Akt
c-fos, c-jun,
Gene expression
Pain sensation
Glycolysis, glycogen synthesis,
protein synthesis
Apoptosis, gluconeogenesis

molecules such as glutamate, acetylcholine and dopamine through decreased calcium influx [76, 79, 80], and thus is attributed with antinociceptive effects. Activation of A_1R not only inhibits adenylyl cyclase, but it can also activate phospholipase C (PLC) resulting in cleavage of phosphatidylinositol bisphosphate (PIP_2) and concurrent generation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). Additionally, activation of A_1R is associated with inhibition of P-, Q- and N-type Ca^{2+} channels, and activation of potassium (K^+) channels. [77]

$A_{2A}R$ is predominantly located on GABAergic striatopallidal neurons in the cortex, as well as in immune tissues such as the spleen, thymus and on leukocytes. Activation of this receptor results in activation of adenylyl cyclase, resulting in increased cAMP levels, and can also promote release of neurotransmitters such as glutamate, leading to increased excitatory signaling. As such, activation of $A_{2A}R$ is attributed with increased pain sensation. $A_{2B}R$ shows low levels of expression in many different tissues, including neuronal tissues such as the brain and spinal cord. Activation of $A_{2B}R$ is associated with activation of adenylyl cyclase and increased cAMP levels, as well as activation of PLC, but this receptor has a low affinity for the endogenous ligand adenosine. A_3R is found primarily in peripheral tissues and has been shown to be expressed on glial cells and neurons. A_3R is linked to inhibition of adenylyl cyclase and activation of PLC. [80]

Adenosine receptors have been the target of a variety of therapeutic interventions, and several agonists and antagonists have been identified to pharmacologically manipulate activation or inhibition of adenosine receptors, respectively. Use of these selective agonists and antagonists has aided in the discovery signaling pathways that may be affected following injury to the nervous system, allowing for the development of novel therapeutics that could be used to

improve clinical outcomes. Agonists selective for A_1R have been generated through substitution at specific sites in the adenine nucleoside. For example, substitution at the N^6-position lead to the creation of N^6-cyclopentyladenosine (CPA), a highly selective and potent A_1R agonist. Further modification of this structure with chlorine lead to the development of a slightly more potent agonist, 2-chloro-CPA (CCPA). Caffeine and xanthine moieties, such as theophylline, are nonselective adenosine receptor antagonists. Modification of xanthines has led to the development of selective adenosine receptor antagonists, such as 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), selective for A_1R [81, 82]. Interestingly, caffeine antagonizes both A_1R and $A_{2A}R$, and the stimulatory effects of caffeine have recently been shown to be mediated through $A_{2A}R$ [83].

Peripherally, activation of the adenosine receptor has numerous effects on the cardiovascular system. Intravenous (i.v.) infusion of adenosine lowers heart rate and atrial contractility and is used to treat patients with paroxysmal supraventricular tachycardia, but caution is advised as this has been associated with the development of hypotension. Adenosine is released following ischemia, and activation of A_1R following myocardial infarction may prove to be beneficial to cardiomyoctes by diminishing ischemia-reperfusion induced injury [84, 85]. In the central nervous system, activation of A_1R is associated with inhibition of nociceptive signaling, presumably through inhibition of glutamate release from spinal interneurons. Indeed, mice lacking A_1R ($A_1R^{-/-}$) show increased nociceptive responses to heat in the tail flick assay and following carrageenan-induced inflammatory pain. Following intrathecal (i.t.) administration of the A_1R agonist, N^6-(*R*-phenylisopropyl)adenosine (R-PIA), $A_1R^{-/-}$ mice showed blunted recovery in thermal hyperalgesia compared to WT mice, suggesting that

analgesic adenosine analogues exert their antinociceptive effects through centrally located A_1R's [79, 86].

Although animal models showing the beneficial antinociceptive effects of adenosine seem promising, translating the immense amount of knowledge into therapies for human neuropathic pain has remained challenging. Human studies investigating adenosine as a potential analgesic have shown limited efficacy. In a small-scale tolerability study, patients receiving i.t. adenosine (500 µg or 1000 µg) reported decreased spontaneous and evoked pain during infusion, as well as increased withdrawal thresholds to mechanical stimuli. Transient low back pain was reported in a few patients, but this resolved within 30 min following administration of adenosine [87]; headache has also been reported following i.t. adenosine [88]. In a larger placebo-controlled study, systemic (i.v.) infusion of adenosine (40-50 µg/kg/min for 60 min) resulted in increased tactile pain thresholds as well as patient-reported improvements in overall general pain sensation. Interestingly, three patients reported improvement in pain symptoms for several hours, and two patients reported complete pain relief for over six months [89]. Another study comparing the efficacy of i.t. versus i.v. adenosine found that i.t., but not i.v., adenosine decreased the area of allodynia and reduced evoked pain in response to mechanical stimuli, but that neither were effective in reducing ongoing or spontaneous pain [88].

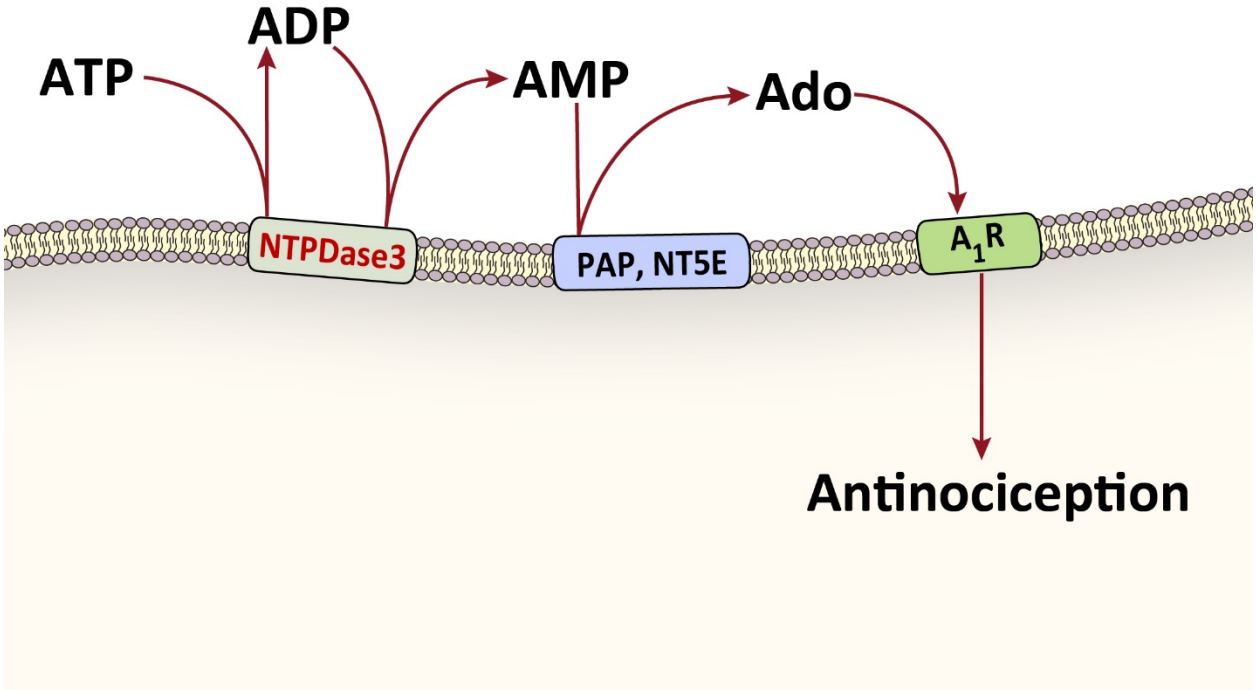
Ectonucleotidases

As mentioned previously, one of the major sources of extracellular adenosine is through hydrolysis of extracellular ATP by specialized enzymes termed ectonucleotidases. Ectonucleotidases belong to a class of ecto-enzymes, which have their catalytic domain oriented

towards the outside of the cell. Studies investigating the role of ecto-enzymes in physiological processes has made significant strides over the last few decades as the technologies for analysis have developed. These enzymes regulate diverse physiological processes, acting to mitigate ATP-induced pro-nociceptive signaling by hydrolyzing ATP into the analgesic small molecule adenosine (Fig. 4). The role of ectonucleotidases in pain signaling, specifically related to termination of pro-nociceptive signaling through hydrolysis of ATP, has gained attention in recent years. Though the contribution of ectonucleotidases to the development of painful diabetic neuropathy remains largely unknown, two specific ectonucleotidases (discussed in detail below), have been shown to mediate antinociceptive signaling in inflammatory and neuropathic pain conditions.

Generation of adenosine is accomplished by the step-wise degradation of ATP by specific ectonucleotidases. Ectonucleoside triphosphate diphosphohydrolases (E-NTPDases; CD39 family) hydrolyze nucleotide triphosphates, such as ATP, and nucleotide diphosphates, such as ADP, into AMP by cleaving phosphodiesterase bonds. E-NTPDases have short N- and C-terminal domains, two transmembrane domains, and a large extracellular glycosylated catalytic domain. The molecular mass of this protein is predicted around 70-80 kDa. There are eight recognized E-NTPDases, and four have been shown to be expressed on the cell surface (NPTDase1, 2, 3 and 8) [90, 91]. NTPDase1 (CD39) can be found in vascular endothelium, and has been shown to have a role in preventing platelet aggregation by hydrolyzing the potent aggregator ADP [92]. Additionally, NTPDase3 has recently been shown to be expressed on

Figure 4. Ectonucleotidase mediated hydrolysis of ATP into adenosine. Hydrolysis of extracellular ATP is mediated by specialized enzymes called ectonucleotidases. E-NTPDases hydrolyze ATP and ADP into AMP. NTPDase3 has been identified in the dorsal horn of the spinal cord and the DRG on a similar neuronal population as NT5E and PAP. NT5E are responsible for hydrolyzing AMP into adenosine, which can then go on to activate A_1R resulting in antinociceptive effects.


ATP
ADP
AMP
Ado
NTPDase3
PAP, NT5E
A1R
Antinociception

putative nociceptors in the DRG and dorsal horn of the spinal cord, and is associated with NT5E expression (see below). Further, NTPDase immunoreactivity was seen in the epidermis of the skin, as well as associated with Merkel cells and keratinocytes, suggesting that NTPDases may play a role in regulating the peripheral nociceptive environment [93].

Ecto-5'-nucleotidase (NT5E; CD73 family) hydrolyzes 5'-AMP into adenosine, and this is the rate-limiting step in the termination of ATP-mediated signaling. The CD73 family of ectonucleotidases are glycophosphatidylinositol (GPI) anchored proteins, and, therefore, do not possess any membrane spanning domains. These proteins are also glycosylated and form dimers that are held together through non-covalent bonds [94]. Interestingly, NTPDase1 and NT5E are highly co-expressed in the brain, which could represent an endogenous physiological mechanism to minimize injury resulting from trauma that causes mass release of ATP and other pro-inflammatory mediators. Moreover, NTPDase1 and NT5E are co-expressed on $CD4^+/CD25^+/Foxp3^+$ T regulatory cells and likely function to inhibit T cell proliferation and cytokine secretion by hydrolyzing ATP, and subsequently, AMP into the anti-inflammatory mediator adenosine [95, 96].

There are several well-known examples of ectonucleotidases that perform important biological functions, such as cholinesterase which is responsible for terminating cholinergic signaling in nerve synapses; enzymes present in the brush borders of the intestines that aid in the digestion of food and nutrients; and the angiotensin converting enzyme (ACE) which is the target of ACE inhibitors used to treat hypertension [90]. Recent evidence has highlighted the role that NT5E and another ectonucleotidase, prostatic acid phosphatase (PAP), play in exerting

antinociceptive effects in the setting of neuropathic and inflammatory pain. Akkina et al. demonstrated altered enzymatic activity in the dorsal horn of the spinal cord as early as four weeks following induction of diabetes [97]. The enzyme thiamine monophosphatase (TMPase) hydrolyzes the vitamin B_1 derivative thiamine monophosphate (TMP), and, historically, this enzyme was used to identify small diameter, nonpeptidergic neurons [98]. However, once IB4 was identified as an easier-to-use marker of the same neuronal population, use of TMPase as a marker of nociceptive neurons faded [99]. Despite the important role this enzyme played in anatomical identification of nociceptive neurons, the physiological function of TMPase remained largely unknown. It was not until 2008, when TMPase was identified as the membrane bound form of PAP [100], that a putative role for this enzyme was put forth.

PAP has been studied for over 70 years in the prostate cancer field. It was originally identified (and is still used) as a serum maker for prostate cancer metastases, but the physiological function remained elusive [101]. In 2007, Quintero et al. were the first to identify the presence of PAP in tissues other than the prostrate [102]. Two splice variants of PAP were identified, a membrane-bound form (TM-PAP) and a secretory form (S-PAP), and TM-PAP was found to be highly expressed in the thymus, lung, spleen, kidney, thyroid and salivary gland, and to a lesser extent in the brain and prostate. In light of this discovery, Zylka and colleagues set out to identify the molecular identity of TMPase.

Using a series of molecular and genetic techniques and animal models of pain, Zylka et al. were able to determine that TMPase was the recently discovered TM-PAP [100]. Transfection of HEK293 cells with TM-PAP showed TMPase activity, and genetic deletion of exon 3 in mice,

which causes loss of PAP catalytic activity ($PAP^{-/-}$ mice), resulted in a loss of TMPase histochemical staining in the DRG and spinal cord. Further characterization of PAP expression in the DRG and spinal cord revealed that it is primarily expressed on small-diameter, nonpeptidergic neurons, identical to that of TMPase. PAP co-localizes extensively with the nonpeptidergic markers IB4, P2X3, and *Mrgprd*, whereas there is little overlap with TRPV1 and CGRP-containing peptidergic neurons [100, 103].

Given the location of PAP on small-diameter, putative nociceptive neurons, it seemed plausible that PAP may play a role in modulating pain sensation. To elucidate the role of PAP in nociception, the behavioral responses of $PAP^{-/-}$ mice following complete Freud's adjuvant (CFA)-induced inflammatory pain or the spared nerve injury (SNI) model of neuropathic pain were evaluated [100]. $PAP^{-/-}$ mice showed enhanced thermal and mechanical sensitivity in the CFA model of inflammatory pain compared to WT mice, whereas only thermal sensitivity was exaggerated in the SNI model of neuropathic pain. Surprisingly, a single i.t. injection of purified human secretory PAP (hPAP) resulted in long lasting (>3 days) antinociception, causing transient improvement of mechanical allodynia and thermal hyperalgesia in both models. Furthermore, this effect was eliminated in mice lacking functional A_1R ($A_1R^{-/-}$ mice), suggesting that the antinociceptive effects of hPAP are mediated through activation of A_1R, and this is dependent on the ability of PAP to hydrolyze extracellular AMP into adenosine.

Surprisingly, AMP histochemistry in $PAP^{-/-}$ mice did not reveal complete abolishment of AMPase activity in the DRG and spinal cord, suggesting the presence of another enzyme in these tissues that is also capable of hydrolyzing AMP into adenosine [100]. Subsequent work led to the

identification of NT5E as the other primary ectonucleotidase in the DRG and spinal cord that hydrolyzes AMP [104, 105]. Expression of NT5E overlapped extensively with PAP, as well as IB4, *Mrgprd*, and P2X3, and again showed little overlap with CGRP and TRPV1, confirming the presence of NT5E on small-diameter, nonpeptidergic putative nociceptive neurons. Additionally, NT5E, but not PAP, expression was seen in the epidermis of the skin, particularly on *Mrgprd* $^{+}$ neurons and keratinocytes. NT5E$^{-/-}$ mice showed reduced AMPase activity in the DRG and spinal cord, with the remaining AMPase activity attributed to PAP activity [104].

Similar to PAP$^{-/-}$ mice, NT5E$^{-/-}$ mice showed exaggerated thermal and mechanical sensitivity following CFA-induced inflammatory pain, but only heightened thermal sensitivity following SNI-induced neuropathic pain. When administration of i.t AMP failed to produce any behavioral outcomes in NT5E$^{-/-}$ mice, it was hypothesized that adenosine generated by hydrolysis of AMP by any remaining nucleotidases was being converted by adenosine kinase or adenosine deaminase to AMP or inosine, respectively, before it was able to activate A_1R. Thus, the ENT and adenosine kinase inhibitor, 5'-iodotubercidin (ITU), was co-administered with AMP [106]. Subsequently, NT5E$^{-/-}$ mice showed moderate increases in thermal withdrawal latencies compared to WT mice, whereas $A_1R^{-/-}$ mice did not show any changes in withdrawal latencies. Following CFA-induced inflammatory pain, NT5E$^{-/-}$ mice given i.t. AMP + ITU showed blunted recovery of thermal withdrawal latencies compared to WT mice. Collectively, these data suggest that NT5E contributes to AMP hydrolysis *in vivo*, and that the antinociceptive effects are dependent both on the generation of adenosine and subsequent activation of A_1R. [104]

Further evaluation of the effect of i.t. hPAP revealed a possible link between A_1R activation and modulation of TRPV1 activity, which could explain the observed thermal antihyperagesic effects following i.t. administration of hPAP. Activation of TRPV1 has been shown to be modulated by levels of PIP_2, and there are two opposing theories regarding the regulation of TRPV1 by PIP_2. The first suggests the presence of PIP_2 in the membrane inhibits TRPV1 function, and cleavage of PIP_2 by PLC releases TRPV1 from this inhibition [107]. Conversely, the second mechanism suggests that PIP_2 is necessary for TRPV1 function, that cleavage of PIP_2 leads to desensitization TRPV1, and that re-synthesis of PIP_2 is necessary for channel recovery [108]. Later studies indicated that the apparent discrepancy regarding modulation of TRPV1 activity by PIP_2 is dependent upon the concentration of capsaicin used. Strong stimuli (i.e. high capsaicin) results in activation of PLC leading to cleavage of PIP_2 and desensitization of TRPV1, whereas with mild to moderate stimuli PIP_2 exhibits an inhibitory effect on TRPV1 and cleavage of PIP_2 relieves this inhibition [109]. In relation to i.t. hPAP-mediated antinociception, activation of A_1R is associated with activation of PLC. Thus, interventions (such as i.t. hPAP) that increase extracellular levels of adenosine, leading to increased activation of A_1R, may be able to modulate thermal hyperalgesia by activating PLC and cleaving PIP_2, thereby inhibiting TRPV1 channel activity [110].

To further investigate the role of PAP and NT5E in modulating nociceptive circuitry, Zylka and colleagues generated double knockout mice that were deficient in both PAP and NT5E (dKO) [105]. PAP and NT5E have different pH profiles at which they are most effective. PAP is an acid phosphatase and thus has optimal activity at acidic pH, specifically pH 5.6, at which it

can hydrolyze AMP, ADP and ATP. PAP is also able to hydrolyze AMP >> ADP at neutral pH (pH 7.0) [111]. The optimal pH for NT5E-mediated hydrolysis of AMP is pH 7.0 [104]. Double knockout mice exhibited near complete loss of AMP hydrolytic activity at both pH 7.0 and pH 5.6 in small and medium diameter neurons, revealing the PAP and NT5E are the primary enzymes in the DRG and spinal cord responsible for hydrolyzing AMP. Furthermore, the behavioral responses of dKO mice following i.t. AMP + ITU were analogous to those of $A_1R^{-/-}$ mice, providing further evidence that PAP and NT5E are responsible for hydrolyzing AMP into adenosine, and that activation of A_1R is responsible for the observed antinociceptive effects in inflammatory and neuropathic pain models. [105]

Collectively, these data indicate that PAP and NT5E are located on small diameter, putative nociceptive neurons in the DRG, as well as in the central terminals where these sensory nociceptive neurons terminate in the dorsal horn of the spinal cord, placing them in prime location to modulate nociceptive signaling. These enzymes both hydrolyze AMP to generate adenosine and, thus, exert their antinociceptive effects through activation of A_1R. Following activation of A_1R, several downstream signaling pathways are activated, including inhibition of adenylyl cyclase and decreased production of cAMP, as well as activation of PLC leading to cleavage of PIP_2 and possible modulation of TRPV1-mediated thermal hyperalgesia.

Statement of Purpose

In the chapters that follow, the case will be made that diabetes disrupts endogenous activation of the A_1R-mediated antinociceptive pathway. Moreover, *in vivo* studies will show that activation of this pathway can alleviate signs of mechanical allodynia in diabetic mice, despite the effect of diabetes on endogenous antinociceptive signaling. Importantly, these studies are the first to show that diabetes affects the ability of ectonucleotidases to hydrolyze AMP, thus disrupting endogenous production of adenosine and activation of the antinociceptive adenosine A_1 receptor. Under physiological conditions, activation of A_1R by adenosine exerts a tonic inhibitory effect on neurons in the dorsal horn, and disruption of this endogenous antinociceptive pathway leads to a loss of inhibition and a concurrent increase in excitatory glutamatergic signaling [112]. Interference of endogenous activation of A_1R as a consequence of long-standing hyperglycemia and diabetes could be yet another mechanism underlying the development of painful diabetic neuropathy. Indeed, P1 receptor activation (A_1R activation) is often considered to be opposite and counteractive to P2 receptor activation (P2X and P2Y receptor activation).

Currently, there are no medications approved by the FDA that treat the underlying pathogenesis contributing to the development of painful diabetic neuropathy. This highlights the need to develop new therapeutic options for managing painful diabetic neuropathy based on the underlying mechanisms, rather than just symptomatic treatment. Studies contained herein will show that although diabetes disrupts adenosine production, this important antinociceptive pathway can still be targeted to significantly improve mechanical sensitivity in the setting of painful diabetic neuropathy. Furthermore, these studies show that central and peripheral delivery

of pharmacological agents which activate A_1R can alleviate signs of painful diabetic neuropathy, mitigating the potential for systemic adverse effects that are often seen following oral or intravenous drug administration.

In summary, these studies support the hypothesis that diabetes alters endogenous adenosine production through inhibition of enzymatic hydrolysis of AMP, and that activation of A_1R can provide a novel target for treating painful diabetic neuropathy in humans.

CHAPTER 2

Central delivery of A_1R agonists reverse mechanical allodynia in a rodent model of painful diabetic neuropathy

1. Abstract

Painful diabetic neuropathy (PDN) is one of the most common and debilitating complications of diabetes mellitus. The current study used a mouse model of PDN to test the hypothesis that the antinociceptive adenosine A_1 receptor (A_1R) is a potential therapeutic target for the treatment of PDN. Seven weeks following i.p. injection of streptozocin (STZ), diabetic mice displayed a significantly decreased ability of ectonucleotidases in the dorsal root ganglia to hydrolyze adenosine monophosphate (AMP) into adenosine, as well as a significant decrease in hindpaw mechanical withdrawal threshold. Intrathecal injection of the ectonucleotidase prostatic acid phosphatase was ineffective at alleviating mechanical allodynia; however, co-administration of AMP or adenosine with the adenosine kinase inhibitor, 5-iodotubercidin, transiently increased mechanical withdrawal thresholds in diabetic mice to nondiabetic levels. Intrathecal administration of the direct A_1R agonist, N^6-cyclopentyladenosine (CPA), reinforced that this behavioral outcome was the result of A_1R activation. This study demonstrates that diabetes affects endogenous adenosine production, but that this important antinociceptive pathway can still be activated via central delivery of A_1R agonists, resulting in an improvement in mechanical sensitivity in diabetic mice.

2. Introduction

Diabetic neuropathy is one of the most common and debilitating symptoms of diabetes, and up to 30% of patients with diabetic neuropathy experience symptoms of pain that negatively impact their quality of life [17, 113]. Painful symptoms can include spontaneous sensations such as burning or tingling (paresthesias), increased sensitivity to a normally innocuous stimuli (allodynia), or heightened sensitivity to an already painful stimulus (hyperalgesia). Several mechanisms are thought to contribute to the development of painful neuropathy, including damage, sensitization, altered trophic support, or spontaneous activity of peripheral pain nerves; central sensitization of dorsal horn neurons; spinal microglial activation; alterations in descending pain control; and altered molecular signaling within the peripheral and central nervous system [5, 113-115]. Unfortunately for these patients, few treatment options exist that provide symptomatic relief and most are either ineffective or have intolerable side effects [27, 115-117].

Pain sensation from the periphery is conveyed through thinly myelinated Aδ-fibers, and unmyelinated C-fibers. As discussed in Chapter 1, these fibers typically have a high threshold for activation, and, thus, primarily convey noxious sensations in the form of heat, cold, mechanical or chemical stimuli. Specific channels and receptors on these nociceptive neurons are responsible for converting these environmental cues into electrical signals that are processed by the central nervous system. Activation of certain receptors results in inhibition of pain transmission, and these receptors can be considered antinociceptive, such as the adenosine A_1 receptor (A_1R). A_1R is a G-protein coupled receptor that is activated by adenosine, and is expressed in both the

peripheral and central nervous system. Activation of this receptor has been implicated in mitigating pain sensation in a variety of neuropathic pain conditions, primarily through inhibition of excitatory signals to higher processing centers [112, 118-120].

Extracellular adenosine is generated through two main sources: hydrolysis of extracellular AMP by ectonucleotidases, or though release of intracellular adenosine by equilibrative nucleotide transporters in the cell membrane. Two ectonucleotidases, prostatic acid phosphatase (PAP) and ecto-5'-nucleotidase (NT5E), have recently been identified on putative nociceptive neurons, and are believed to mitigate pain sensation by hydrolyzing AMP into adenosine and activating A_1R (covered in detail in Chapter 1). However, the effect of diabetes on ectonucleotidase expression and function remained unknown. Here, we show that diabetes does indeed affect ectonucleotidase activity, primarily on small- and medium-diameter neurons in the DRG, which are associated with transmitting pain sensation from the periphery to the central nervous system.

Current treatment options for painful diabetic neuropathy are limited primarily to symptomatic treatment and come with significant risk of side effects and questionable efficacy. None of the currently available treatment options for painful diabetic neuropathy are aimed at treating the pathogenesis underlying development of the disease [17]. Furthermore, very few studies have ever investigated the potential of A_1R-mediated antinociception as a potential novel treatment option for painful diabetic neuropathy, and most studies evaluating this outcome investigated the effects on thermal and chemical nociception. As such, we sought to determine if A_1R activation could modulate pain sensation in diabetic mice that display signs of mechanical

allodynia, a symptom often associated with painful diabetic neuropathy. Indeed, we were able to show that central activation of A_1R was able to reverse mechanical allodynia in diabetic mice

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3. Experimental Procedures

Animals

All experiments were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. Inbred male A/J mice were obtained from Jackson Laboratories at 7 weeks of age, and were induced with diabetes at 8 weeks of age. All mice were housed in the Laboratory Animal Resources building, had *ad libitum* access food and water, and maintained on a 12 hour light/dark cycle. All mice were euthanized at 16 weeks of age.

Streptozocin induction of diabetes

Male A/J mice were injected with Streptozocin (STZ; Sigma, St. Louis, MO) at 8 weeks of age to induce type 1 diabetes. Mice were fasted for 3 hours before and after injections, for a total fasting time of 6 hours. Injections were spread over 2 days, with the first dose (day 1) at 85 mg/kg and the second dose (day 2) at 65 mg/kg. Solutions were made fresh immediately prior to injection and STZ was dissolved in ice cold 10 mM sodium citrate buffer with 0.9% NaCl at pH 4.5, and then filter sterilized. Control, nondiabetic mice were injected with sodium citrate buffer only. Animals that did not reach hyperglycemia (blood glucose >230 mg/dL) within one week following initial injections were re-injected with either 85 mg/kg STZ (blood glucose <180 mg/dL) or 65 mg/kg STZ (blood glucose 181-230 mg/dL). Mice that failed to reach hyperglycemia were excluded from the study.

Blood glucose measurements

Weight and blood glucose measurements (glucose diagnostic reagents; Sigma) were collected one week after initial injection and every week thereafter. Mice were fasted for 3 hours prior to collection of blood collection from the tail. Mice were considered diabetic when blood glucose levels were >230 mg/dL.

Behavioral analysis

Prior to behavioral testing, mice were acclimated to the behavior facility and equipment for a minimum of 2 days. On test days, mice were placed in the behavior facility and allowed to acclimate to the environment for at least 30 minutes. Mice were then acclimated on the behavior apparatus for 20 to 30 minutes prior to initiating testing. Mice were placed in individual clear plastic cages on top of a wire mesh grid that allowed access to their hind paws for the duration of the analysis. Mechanical withdrawal thresholds using von Frey monofilaments were measured weekly to track progression of neuropathy phenotype using the up-down method to determine fifty percent withdrawal thresholds [121]. Tests for mechanical sensitivity following intrathecal (i.t.) injections were performed at 30 min, 1.5 hours, 3 hours and 5 hours following injection; mechanical sensitivity following injection of i.t. purified human PAP (hPAP) was evaluated at 24, 48, 72 and 120 hours post-injection.

Drugs and Drug Administration

Adenosine (Ado), AMP and CPA (direct A_1R agonist) were purchased from Sigma; hPAP was graciously provided by Dr. Mark Zylka (University of North Carolina). 5'iodotubercidin (ITU; adenosine kinase inhibitor) was purchased from Enzo Life Sciences. AMP, Ado, and CPA were dissolved in 0.9% saline at pH 7.4. AMP was delivered at a

concentration of 200 nmol/10 µl [104]; Ado and CPA were delivered at 10 nmol/10 µl [122, 123]; hPAP was dissolved in saline and delivered at a dose of 250 mU/10 µL [100]. ITU was co-administered with Ado or AMP at a concentration of 5 nmol/10 µL [124]. Intrathecal injections (hPAP, AMP + ITU, Ado + ITU, CPA) were performed using a 28 gauge, 8 mm insulin syringe (BD Biosciences, San Jose, CA) using the direct lumbar puncture method between L5-L6 [125].

Tissue Preparation

Mice were overdosed with inhaled isoflurane, injected with 150 µl of heparin (BD Biosciences) into the ventricle, and then transcardially perfused with 4% paraformaldehyde (in 1 x PBS, pH 7.4). Lumbar spinal cord and dorsal root ganglia (DRG, L4-6) were dissected, post-fixed in 4% paraformaldehyde for 1 hour, and cryo-protected overnight in 30% sucrose (w/v in 1 x PBS). Tissues were frozen in OTC and stored at -20°C until sectioning. Spinal cord tissue was serially sectioned at 10 µm and DRG were sectioned at 8 µm; tissues were mounted on Suprafrost slides (Fisher Scientific, Chicago, IL) and stored at -20°C until used.

Immunohistochemistry

Slides were brought to room temperature in a humidity chamber, outlined with PAP Pen (Research Products International, Mt. Prospect, IL) to create a hydrophobic barrier, and blocked for one hour at room temperature in a pre-incubation solution (PIS) consisting of Superblock (Thermo Scientific, Rockford, IL), 1.5 % normal goat serum, 0.5% porcine gelatin and 0.5% Triton X-100. Primary antibodies were diluted in PIS and incubated overnight at 4°C. Slides were washed twice in 1 x PBST, and then incubated with secondary antibody for 1 hour at 4°C in 1 x PBST and Superblock in a 1:1 ratio. Slides were washed twice in 1 x PBS, coverslipped and imaged using fluorescence microscopy. Primary antibodies included: chicken anti-PAP (1:4,000;

Aves Labs, Tigard, OR), isolectin IB4 Alexa Fluor 488 conjugate (1:50; Invitrogen, Carlsbad, CA), and rabbit-anti-TRPV1 (1:1000; Calbiochem). Secondary antibodies included: Donkey anti-chicken-555 (1:2000; Jackson) and donkey anti-rabbit-647 (1:2000; Invitrogen). The overall percent positive neurons in the DRG was determined by counting the number of PAP-positive neurons and the number of neurons overall in both nondiabetic and diabetic mice. Spinal cord slices were evaluated by placing regions around the medial and lateral one third of PAP-positive bands in the dorsal horn of the spinal cord in both nondiabetic and diabetic mice. Images were analyzed using NIS Elements software (Nikon Corporation).

Enzyme Histochemistry

Ectonucleotidase activity (PAP and NT5E) was quantified using enzyme histochemistry. Sections of spinal cord or DRG were incubated in trisma-maleate (Sigma) buffer with 8% sucrose (w/v; TMBS) at pH 7.4 or pH 5.6 for 30 minutes. Tissues were then incubated in TMBS solution containing 2.4 mM lead nitrate and either 3 mM AMP (DRG, pH 7.4) or 0.3 mM AMP (DRG, pH 5.6; spinal cord pH 7.4 and pH 5.6) for 2-3 hours. Tissues were developed in 0.5% ammonium sulfide solution, rinsed three times in TMBS, coverslipped and imaged on a light microscope. Positive neurons were identified by the presence of a dark brown lead phosphate precipitate. DRG soma areas were calculated using NIH Image J software by tracing the border of all positive and negative neurons. At pH 7.4, sections from 7 nondiabetic and 7 diabetic mice were counted, and at pH 5.6 sections from 9 nondiabetic and 8 diabetic mice were counted. A minimum of 500 neurons per animal were evaluated. Hydrolysis of AMP in the spinal cord was analyzed using NIS Elements software (Nikon Corporation) by placing regions of interest around the medial and lateral 1/3 of each band of precipitate in the dorsal horn. At pH 7.4, sections from

7 nondiabetic and 7 diabetic mice were, and at pH 5.6, sections from 5 nondiabetic and 7 diabetic mice were counted. A minimum of 5 dorsal horns per animal were evaluated.

Statistical analysis

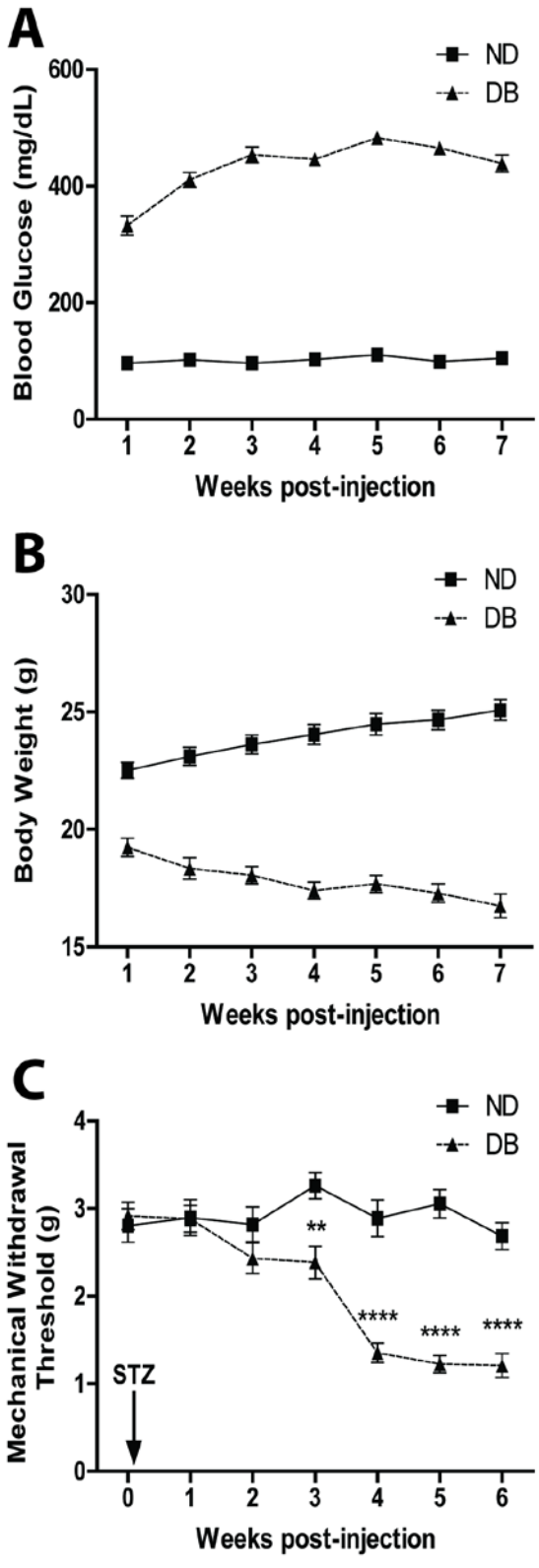
Results were analyzed using the SPSS Statistics 20 software (IBM). Student's t-tests and two-way repeated measures analysis of variance (2-way RM-ANOVA) with Fisher's least significant difference (LSD) post-hoc analyses were performed, as denoted in the manuscript. A *p*-value less than 0.05 was considered significant. All data are presented as mean ± S.E.M.

4. Results & Figures

Diabetic A/J inbred mice develop mechanical allodynia.

Fasting blood glucose and weight measurements were taken weekly following STZ injection. Mice that received STZ had an average fasting blood glucose level of 432.8 +/- 18.7 mg/dL as early as one week post-STZ injection, whereas control mice had an average fasting blood glucose level of 101.7 +/- 1.9 mg/dL (Fig. 5A). Simultaneously, diabetic mice failed to gain weight compared to their nondiabetic counterparts (Fig. 5B). All mice underwent weekly behavioral testing to quantify changes in hind paw mechanical sensitivity. Beginning 3 weeks post- STZ injections, withdrawal threshold was significantly decreased in diabetic mice compared to nondiabetic mice (Fig. 5C). Withdrawal thresholds in diabetic mice reached their lowest levels at four weeks post-injection, and remained consistently decreased compared to nondiabetic counterparts for the duration of the study.

Figure 5. Diabetic mice develop hyperglycemia and mechanical allodynia. A) Blood glucose measurements of diabetic (DB) mice were significantly elevated at all time points beginning 1 week post-STZ injection ($p < 0.0001$ vs. nondiabetic [ND] at all time points; 2-way RM-ANOVA with LSD post-hoc analysis). B) Diabetic mice failed to gain weight, unlike their ND counterparts ($p < 0.0001$ vs. ND at all time points; 2-way RM-ANOVA with LSD post-hoc analysis). C) Diabetic mice displayed a significantly decreased mechanical withdrawal threshold beginning 3 weeks post-STZ injection that persisted throughout the duration of the study (** $p < 0.0001$ vs. ND, 2-way RM-ANOVA with LSD post-hoc analysis). n = 28 – 33 mice per group.


A
ND
DB
Blood Glucose (mg/dL)
600
400
200
0
1
2
3
4
5
6
7
Weeks post-injection
B
ND
DB
Body Weight (g)
30
25
20
15
1
2
3
4
5
6
7
Weeks post-injection
C
ND
DB
Mechanical Withdrawal Threshold (g)
4
3
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1
0
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STZ
0
1
2
3
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5
6
Weeks post-injection

Diabetes alters the ability of ectonucleotidases to hydrolyze AMP in the DRG

Immunohistochemistry was used to confirm protein expression of PAP and NT5E on nonpeptidergic neurons in the DRG and spinal cord. PAP and NT5E expression overlapped extensively with the nonpeptidergic neuronal marker isolectin B4 (IB4) in both the DRG (Fig. 6A-F) and spinal cord (Fig. 7A-F). There was minimal to no overlap between PAP or NT5E with TRPV1, which is primarily expressed on peptidergic neurons in the DRG (Fig. 6G-L) or the spinal cord (Fig. 7G-L). These results are in line with previous reports of PAP and NT5E expression in these tissues [100, 103, 104]. PAP expression was evaluated in nondiabetic and diabetic mice to determine the effect of diabetes on enzyme expression. No change in the overall percentage of positive neurons that express PAP was evident in the DRG (Fig. 8A). In the spinal cord, central termination patterns of nociceptors are arranged such that axons from the distal limbs terminate in the medial portion of the dorsal horn and axons from more proximal tissues terminate in the lateral portion of the dorsal horn [126]. There was no change in overall expression of PAP protein between nondiabetic and diabetic mice in the medial portion of spinal cord (Fig. 8B).

Figure 6. PAP and NT5E are expressed in lumbar DRG neurons. PAP (A, G) and NT5E (D, J) are expressed primarily on small-diameter neurons in the DRG, which also express the non-peptidergic neuronal marker, IB4 (B, E). Merged images (C, F) show extensive overlap between the two markers. TRPV1 (H, K) is primarily expressed on peptidergic neurons in the DRG. Merged images (I, L) between TRPV1 and PAP or NT5E, show minimal overlap between the two markers. Arrowheads point to positive neurons that overlap in merged images. Arrows point to the same neuron throughout the series of images.

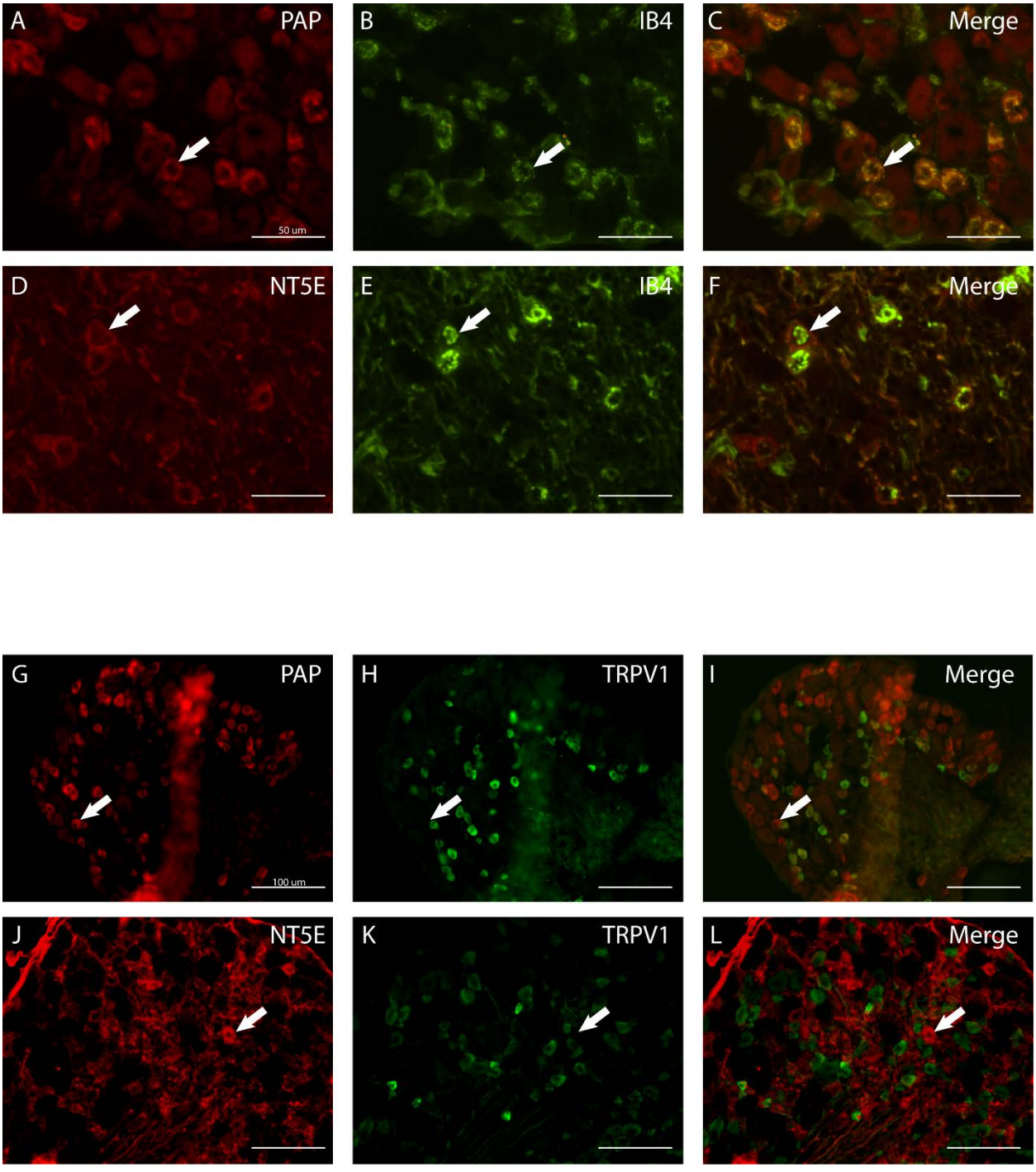

A
PAP
50 um
B
IB4
C
Merge
D
NT5E
E
IB4
F
Merge
G
PAP
100 um
H
TRPV1
I
Merge
J
NT5E
K
TRPV1
L
Merge

Figure 7. PAP and NT5E are expressed in the dorsal horn of the lumbar spinal cord. PAP (A, G) and NT5E (D, J) are primarily expressed in the dorsal horn of lumbar spinal cord sections. IB4 (B, E) is used as a marker of nonpeptidergic nerve terminals in the spinal cord, which terminate in lamina II of the dorsal horn. Merged images (C, F) show extensive overlap between IB4 and PAP or NT5E. TRPV1 (H, K) is primarily expressed on peptidergic neurons, which terminate in lamina I of the dorsal horn. Merged images (I, L) between TRPV1 and PAP or NT5E, show minimal overlap between the two markers.

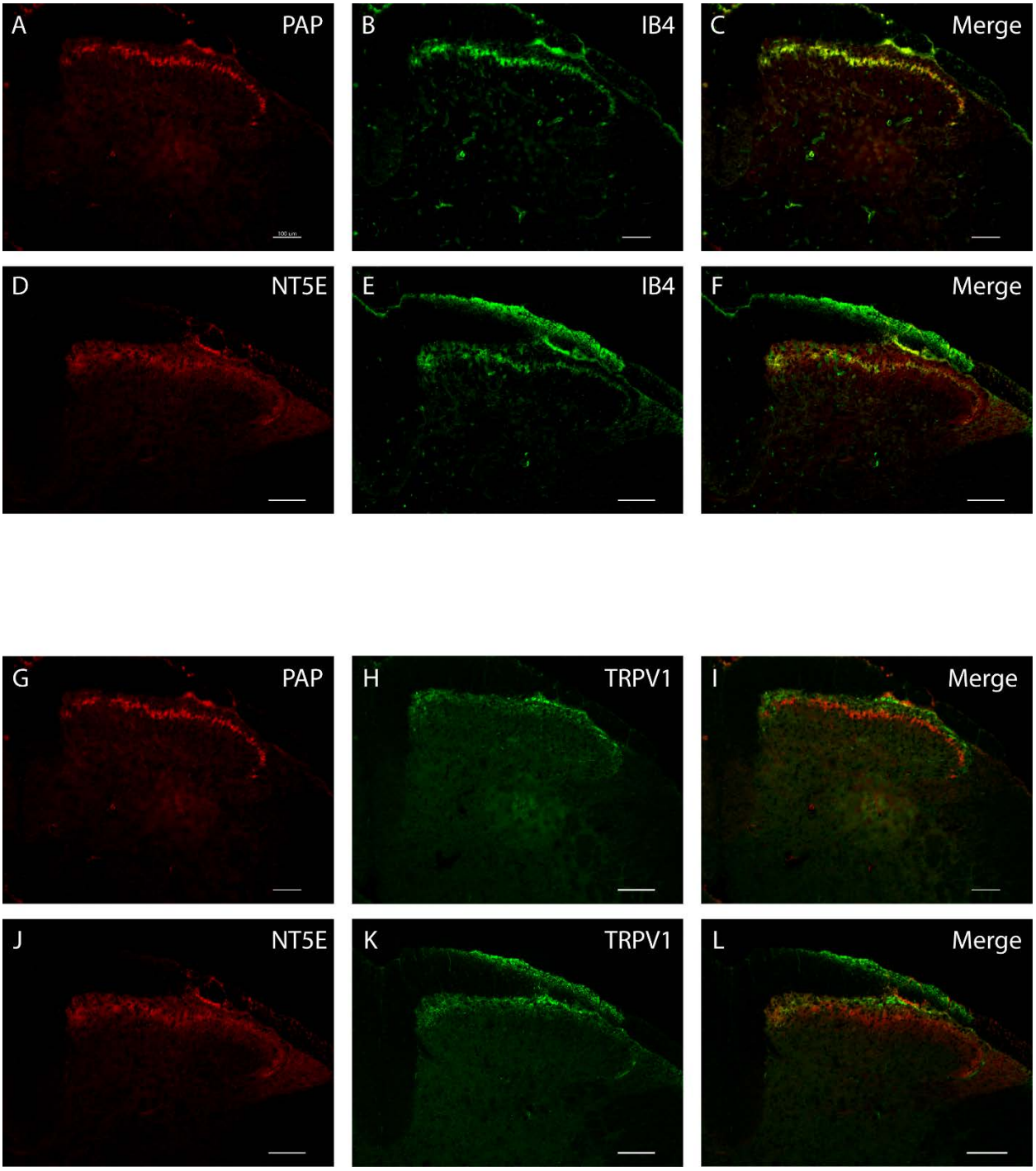
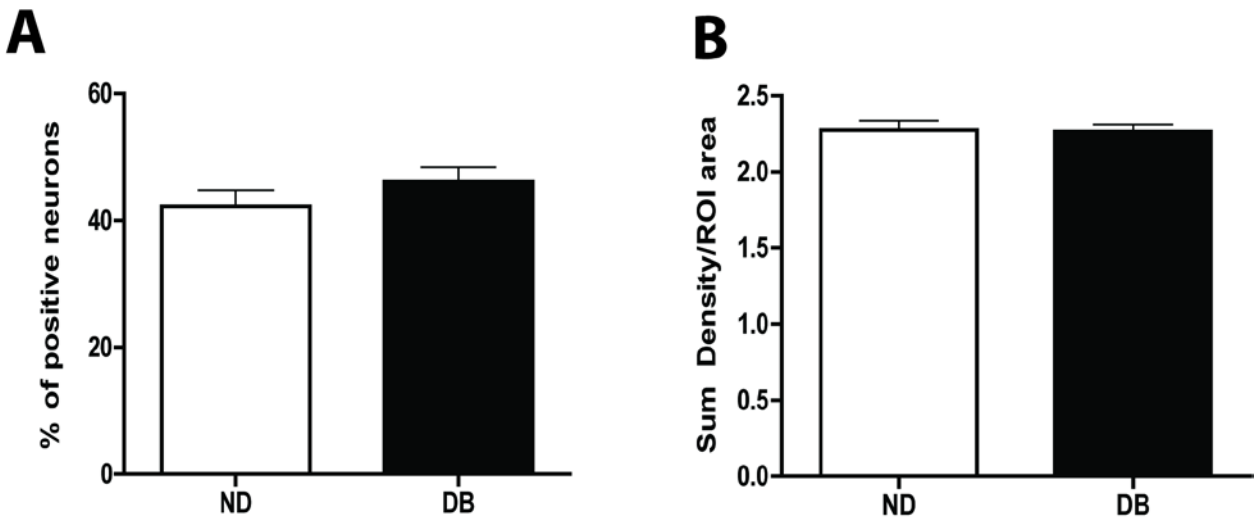

A
PAP
B
IB4
C
Merge
D
NT5E
E
IB4
F
Merge
G
PAP
H
TRPV1
I
Merge
J
NT5E
K
TRPV1
L
Merge

Figure 8. PAP expression is not affected by diabetes in either the DRG or spinal cord. A) The overall percentage of neurons expressing PAP was not significantly affected by diabetes when comparing diabetic (DB) to nondiabetic (ND) mice ($p > 0.05$ vs. ND, t-test); n = 5 – 8 mice per group. B) Expression of PAP in the dorsal horn of the spinal cord of DB mice was also not altered by diabetes ($p > 0.05$ vs. ND; t-test); n = 6 – 8 mice per group.


A
% of positive neurons
60
40
20
0
ND
DB
B
Sum Density/ROI area
2.5
2.0
1.5
1.0
0.5
0.0
ND
DB

Extracellular hydrolysis of AMP is predominantly mediated by PAP and NT5E in the dorsal spinal cord and DRG [100, 104, 105]. NT5E is primarily active near physiological pH (7.0), whereas PAP is the primary enzyme active at acidic pH (5.6) [100, 104]. AMP histochemistry was performed on DRG and spinal cord tissues at pH 7.4 and 5.6 to evaluate the ability of NT5E and PAP, respectively, to degrade AMP in both diabetic and nondiabetic mice. Examples of AMP hydrolysis in the DRG are shown in Figure 9A. In the DRG of diabetic A/J mice, there was a small but statistically significant decrease in the overall percentage of neurons capable of hydrolyzing AMP at pH 7.4 (Fig. 9B), specifically in neurons with small soma areas (<300 μm^2; Fig. 2C), which correspond to neurons within the small-diameter range. At acidic pH, there was no change in the overall percentage of positive neurons in the DRG (Fig. 9B); however there was a significant decrease in the percentage of positive neurons in neurons with soma areas that correspond to the small- (< 300 μm^2) and medium-diameter (301-600 μm^2) range (Fig. 9D). Examples of AMP hydrolysis at pH 7.4 and 5.6 in the dorsal horn of nondiabetic and diabetic mice are shown in Figure 10A. Assessment of enzyme activity in the medial portion of the dorsal horn revealed no significant differences in staining density or patterns between nondiabetic and diabetic mice at physiological or acidic pH (Fig. 10B) [97].

Modulation of nociception by AMP hydrolysis in diabetic mice

Previous studies indicate that a single dose of i.t. hPAP provided long lasting (>3 days) antinociceptive effects in mice that had either inflammatory or neuropathic pain [100, 110, 111]. To determine if hPAP would have the same efficacy in painful diabetic neuropathy, a single injection of i.t. hPAP (250 mU/10 µl) was administered to diabetic and nondiabetic mice. Diabetic mice treated with i.t. hPAP had a significant reversal of their mechanical sensitivity

Figure 9. AMP hydrolysis is decreased in the DRG of diabetic mice. A) Representative images of AMP histochemistry performed in DRG sections from nondiabetic and diabetic mice at pH 7.4 and 5.6. Arrowheads indicate positive neurons; asterisks indicate negative neurons. B) The overall percentage of neurons displaying evidence of hydrolyzed AMP at pH 7.4 was significantly decreased in diabetic (DB) mice, compared to nondiabetic (ND) mice (**p* <0.05 vs. ND, t-test). There was no significant difference in the overall percentage of neurons capable of hydrolyzing AMP at pH 5.6 (**p* > 0.05 vs. ND, t-test). C) The percentage of positive neurons was significantly decreased only within small-diameter (< 300 μm^2) neurons at pH 7.4 (**p* <0.05 vs. ND, 2-way RM-ANOVA with LSD post-hoc analysis). D) At pH 5.6, the percentage of positive neurons was significantly decreased within both the small- (< 300 μm^2) and medium-diameter (301-600 μm^2) neurons (**p* <0.05 vs. ND, 2-way RM-ANOVA with LSD post-hoc analysis). n = 7 mice per group.

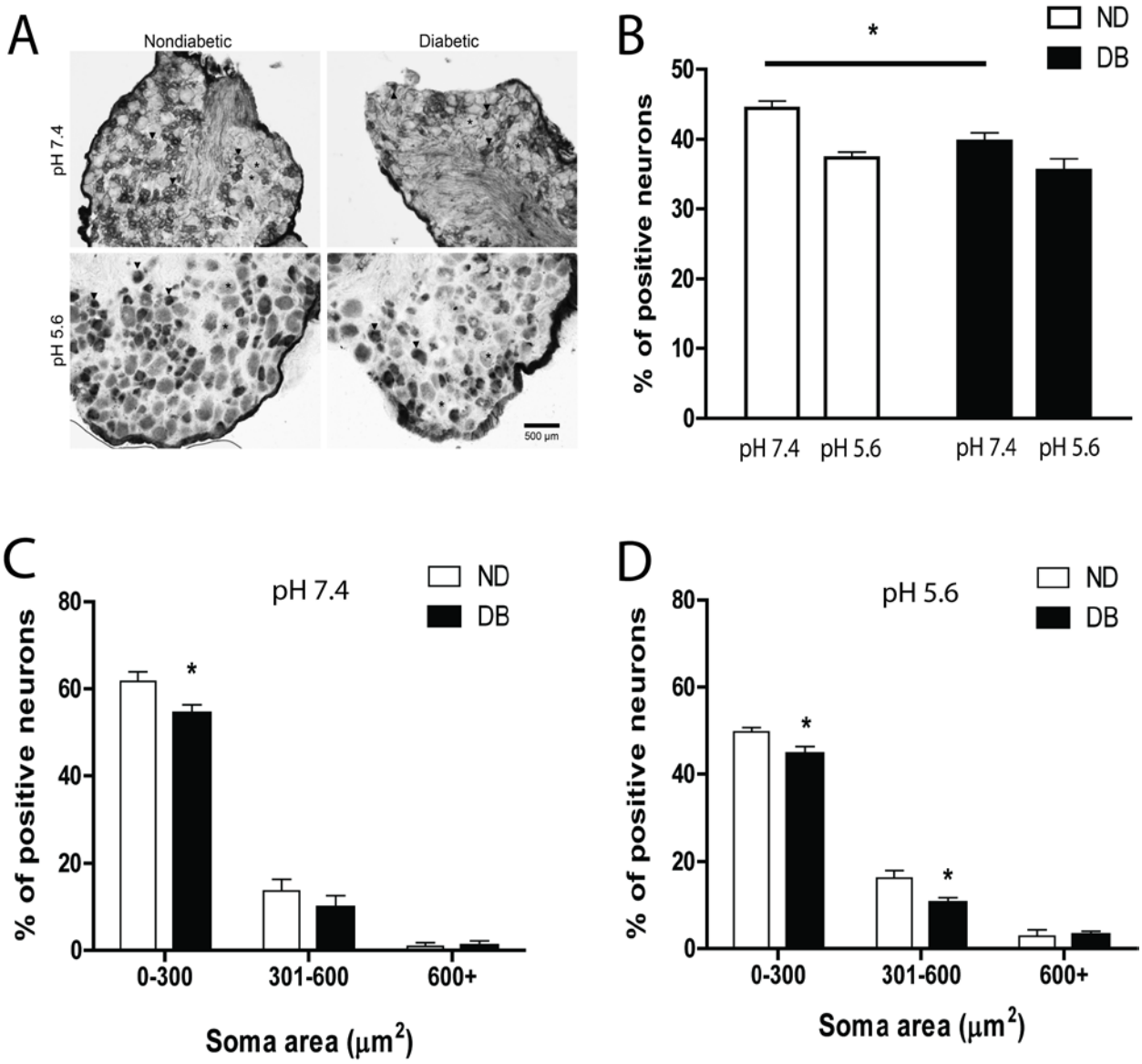
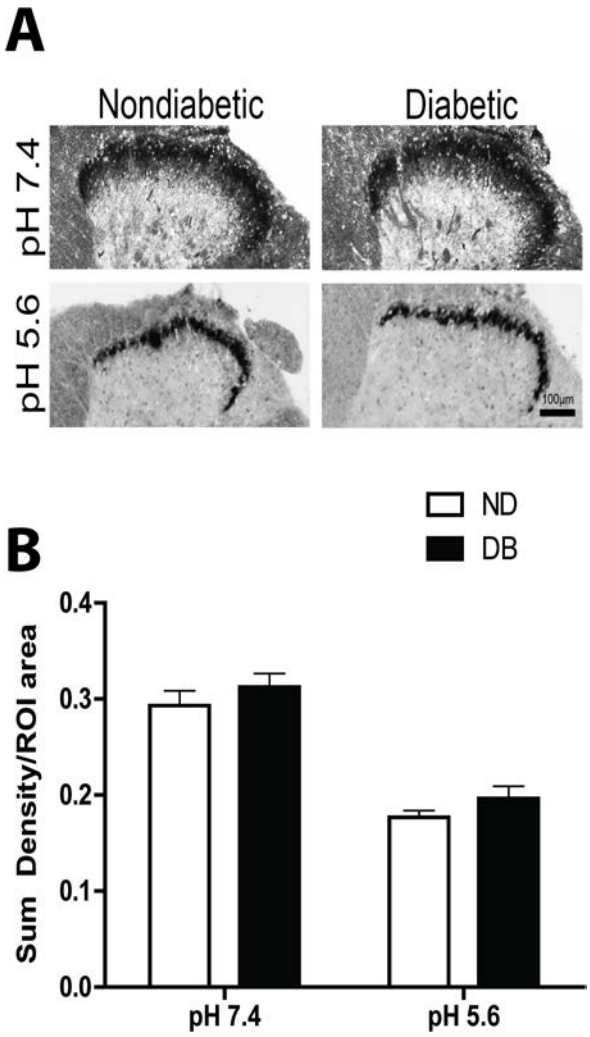

A
Nondiabetic
Diabetic
pH 7.4
pH 5.6
500 µm
B
ND
DB
*
% of positive neurons
50
40
30
20
10
0
pH 7.4
pH 5.6
pH 7.4
pH 5.6
C
pH 7.4
ND
DB
% of positive neurons
80
60
40
20
0
*
0-300
301-600
600+
Soma area (µm²)
D
pH 5.6
ND
DB
% of positive neurons
80
60
40
20
0
*
*
0-300
301-600
600+
Soma area (µm²)

Figure 10. Diabetes does not affect AMP hydrolysis in the dorsal horn of the lumbar spinal cord. A) Representative images of AMP histochemistry in dorsal horn sections from nondiabetic (ND) and diabetic (DB) mice at pH 7.4 and pH 5.6. B) No significant difference in AMP hydrolysis was observed between ND and DB mice at pH 7.4 or at pH 5.6 in the medial portion of the dorsal horn (*$p > 0.05$ vs. ND, t-test). n = 5 -7 mice per group.


A
Nondiabetic
Diabetic
pH 7.4
pH 5.6
100μm
B
ND
DB
Sum Density/ROI area
0.4
0.3
0.2
0.1
0.0
pH 7.4
pH 5.6

at 24 hours post-injection compared to baseline values (p = 0.02, Fig. 11A); however, this this was not significantly different than diabetic vehicle treated mice at 24 hours. There were no significant differences in mechanical withdrawal thresholds between nondiabetic hPAP treated and nondiabetic vehicle treated mice at any time point. At 24 hours post-injection there was no significant difference between nondiabetic and diabetic hPAP treated mice.

Subsequently, i.t. AMP (200 nmol/10 µl) was administered to determine whether the endogenous hydrolytic activity of these enzymes was sufficient to generate adenosine and activate antinociceptive pathways. When given alone, neither AMP (Fig. 12A; or adenosine, Fig. 12B) nor ITU (Fig. 12C) produced any changes in mechanical sensitivity in diabetic. However, when AMP was co-administered with ITU (i.t. AMP + ITU) diabetic mice showed a significant, but transient, reversal of their mechanical sensitivity at 30 min (p = 0.01) compared to pre-treatment withdrawal thresholds (Figs. 11B). The withdrawal threshold of AMP + ITU treated diabetic mice was also significantly different than diabetic vehicle treated mice at 30 min (p = 0.002) following administration. There were no significant differences between nondiabetic AMP + ITU treated mice and nondiabetic vehicle treated mice.

Figure 11. Supplementation of i.t. hPAP and AMP + ITU transiently alleviates mechanical sensitivity in diabetic mice. A) Diabetic (DB) mice administered i.t. hPAP (250 mU/10 µl) showed a significant improvement in mechanical withdrawal thresholds compared to baseline levels after 24 hours (# $p < 0.05$ vs. DB hPAP, 2-way RM-ANOVA with LSD post-hoc analysis); however this was not significantly different from diabetic vehicle treated mice at 24 hours ($p > 0.05$ vs. DB Veh, 2-way RM-ANOVA with LSD post-hoc analysis). n = 9 – 10 mice per group. B) Diabetic mice administered i.t. AMP + ITU (AMP, 200 nmol/10 µl; ITU, 5 nmol/10 µl) showed a transient, but significant, reversal in their mechanical sensitivity at 30 min following injection compared to baseline (## $p = 0.01$ vs. DB AMP + ITU, 2-way RM-ANOVA with LSD post-hoc analysis) and to DB vehicle treated mice (**$p < 0.01$ vs. DB Veh, 2-way RM-ANOVA with LSD post-hoc analysis), which returned to pre-injection withdrawal thresholds after 1.5 hours. n = 9 – 10 mice per group.

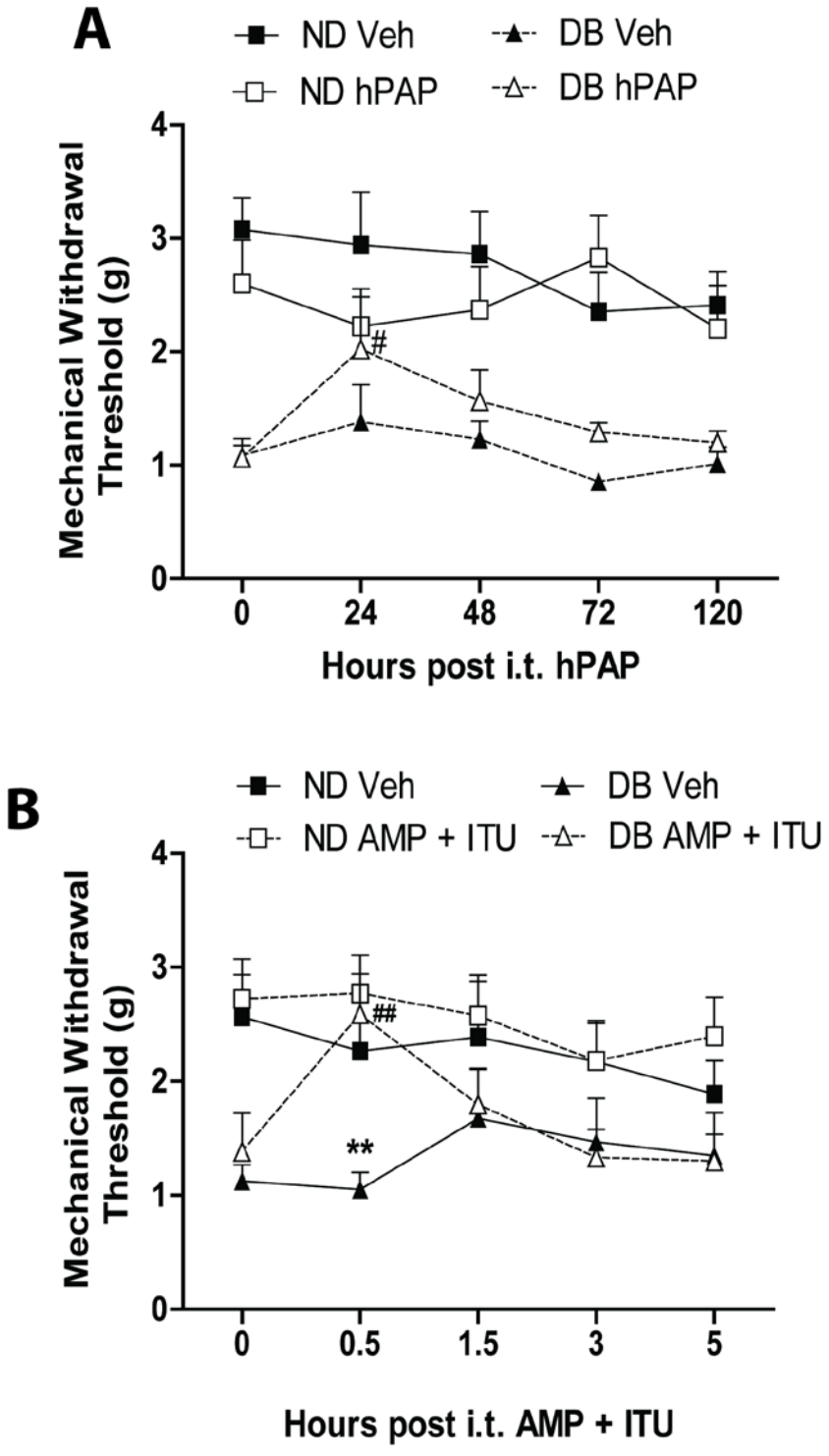
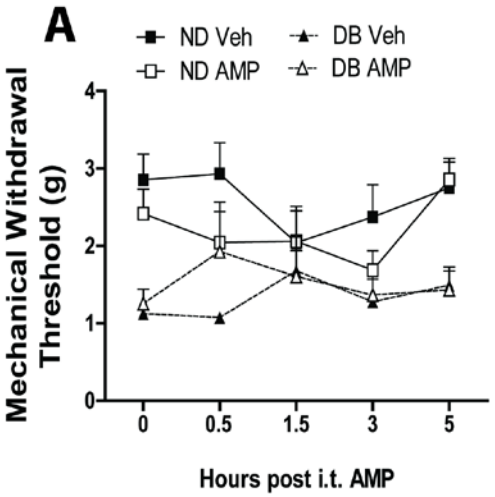
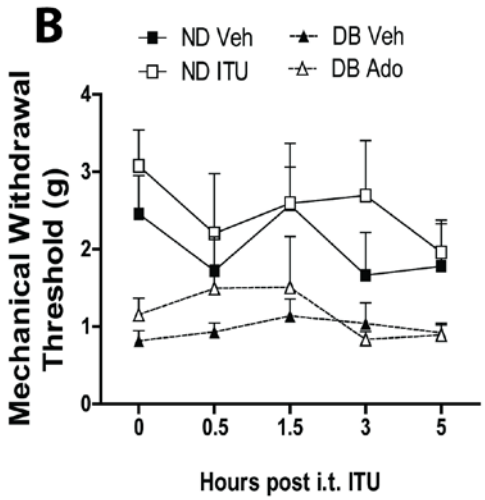
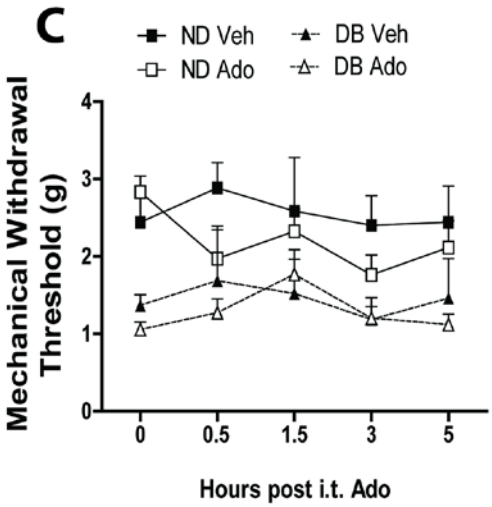

A
ND Veh
DB Veh
ND hPAP
DB hPAP
Mechanical Withdrawal Threshold (g)
0
1
2
3
4
#
0
24
48
72
120
Hours post i.t. hPAP
B
ND Veh
DB Veh
ND AMP + ITU
DB AMP + ITU
Mechanical Withdrawal Threshold (g)
0
1
2
3
4
##
**
0
0.5
1.5
3
5
Hours post i.t. AMP + ITU

Figure 12. Neither i.t. AMP, i.t. Ado nor i.t. ITU alleviate mechanical allodynia when given individually. A) Diabetic (DB) mice administered i.t AMP (200 nmol/10 µl) alone did not show any significant improvement in mechanical withdrawal thresholds compared to baseline (p > 0.05 vs. DB AMP, 2-way RM-ANOVA with LSD post-hoc analysis), or DB-vehicle treated mice (p > 0.05 vs. DB Veh, 2-way RM-ANOVA with LSD post-hoc analysis). n = 5 mice per group. B) DB mice that received only i.t. Ado (10 nmol/10 µl) showed no significant reversal in mechanical allodynia compared to baseline (p > 0.05 vs. DB Ado, 2-way RM-ANOVA with LSD post-hoc analysis), or DB-vehicle treated mice (p > 0.05 vs. DB Veh, 2-way RM-ANOVA with LSD post-hoc analysis). n = 5 mice per group. C) DB mice given i.t. ITU (5 nmol/10 µl) alone did not show any significant improvements in mechanical withdrawal thresholds compared to baseline (p > 0.05 vs. DB ITU, 2-way RM-ANOVA with LSD post-hoc analysis), or DB-vehicle treated mice (p > 0.05 vs. DB Veh, 2-way RM-ANOVA with LSD post-hoc analysis). n = 4 mice per group.


A
ND Veh
DB Veh
ND AMP
DB AMP
Mechanical Withdrawal Threshold (g)
0
1
2
3
4
0
0.5
1.5
3
5
Hours post i.t. AMP


B
ND Veh
DB Veh
ND ITU
DB Ado
Mechanical Withdrawal Threshold (g)
0
1
2
3
4
0
0.5
1.5
3
5
Hours post i.t. ITU


C
ND Veh
DB Veh
ND Ado
DB Ado
Mechanical Withdrawal Threshold (g)
0
1
2
3
4
0
0.5
1.5
3
5
Hours post i.t. Ado

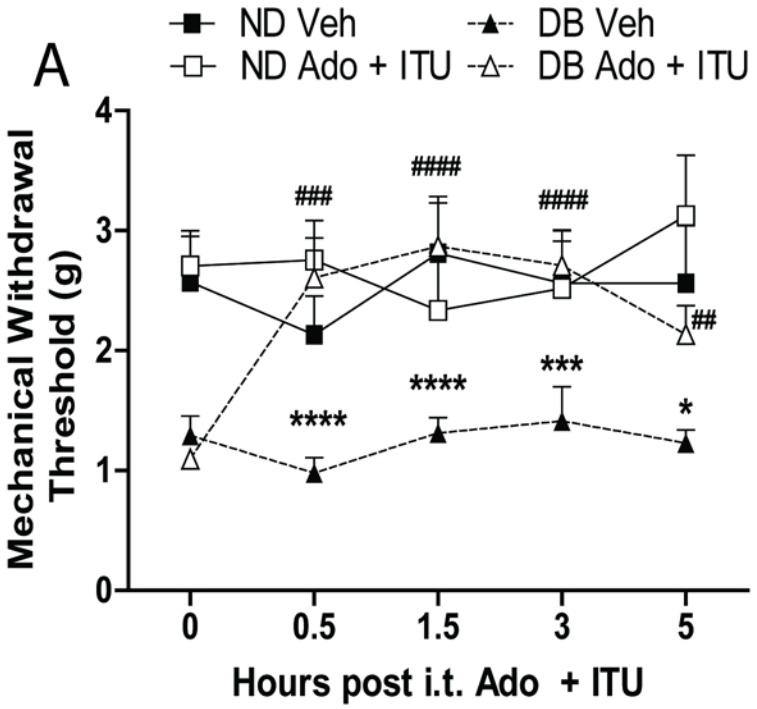
Direct activation of spinal A_1Rs reverses mechanical allodynia.

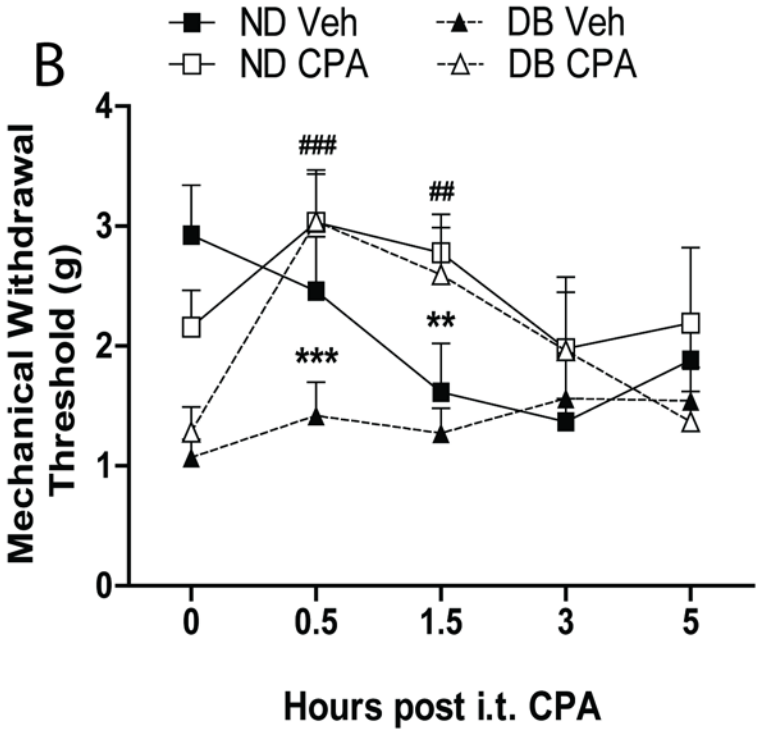
Finally, mechanical sensitivity was assessed following direct activation of A_1R by i.t. administration of adenosine (with ITU, Ado + ITU, 10 nmol/10 µl) or CPA (10 nmol/10 µl), a specific A_1R agonist and stable adenosine analog. Diabetic mice that received Ado + ITU displayed a significant increase in mechanical withdrawal threshold for at least 5 hours compared to baseline ($p < 0.01$), as well as compared to diabetic vehicle treated mice ($p < 0.05$); at 5 hours post-i.t. Ado +ITU, the mechanical withdrawal thresholds of diabetic mice had not yet return to pre-treatment levels (Fig. 13A). There were no significant differences between nondiabetic Ado + ITU treated and vehicle treated mice. Diabetic mice that received i.t. CPA also displayed a significant increase in mechanical withdrawal threshold compared to baseline and compared diabetic vehicle treated mice, lasting through the 1.5h time point ($p < 0.01$; Fig. 13B).

5. Discussion

Painful diabetic neuropathy affects a significant proportion of individuals with diabetic neuropathy, and although many mechanisms have been identified as potential underlying causes of diabetic neuropathy, few of these mechanisms have resulted in the development of a successful therapeutic treatment options to alleviate pain associated with diabetic neuropathy. Rodent models have been key to understanding the pathogenesis of both diabetic neuropathy and painful diabetic neuropathy. Here, we show that inbred male A/J mice develop a robust mechanical allodynia following induction of type 1 diabetes, providing a reliable model for investigating the development of painful diabetic neuropathy as well as potential therapeutic

Figure 13. Central delivery of Ado + ITU or CPA provides long-lasting anti-allodynic effects in diabetic mice. A) Diabetic (DB) mice that received i.t. Ado + ITU (Ado, 10 nmol/10 µl; ITU, 5 nmol/10 µl) showed a significant reversal of mechanical sensitivity compared to baseline (## $p < 0.01$ vs. DB Ado + ITU, 2-way RM-ANOVA with LSD post-hoc analysis) and diabetic vehicle treated mice (* $p < 0.05$ vs. DB Veh, 2-way RM-ANOVA with LSD post-hoc analysis) that lasted over 5 hours, at which time withdrawal thresholds had not yet returned to baseline levels. Withdrawal thresholds of DB mice that received i.t. Ado + ITU were not significantly different from ND Ado + ITU treated mice at any time point. n = 5 – 8 mice per group. B) Intrathecal CPA (10 nmol/10 µl) also significantly reversed mechanical withdrawal thresholds in diabetic mice compared to baseline (## $p < 0.01$ vs. DB CPA, 2-way RM-ANOVA with LSD post-hoc analysis) and diabetic vehicle treated mice (** $p < 0.01$ vs. DB Veh, 2-way RM-ANOVA with LSD post-hoc analysis) for up to 3 hours. Additionally, ND mice that received i.t. CPA had significantly elevated withdrawal thresholds compared to ND vehicle treated mice at 1.5 hours following treatment (^ $p < 0.05$ vs. ND Veh; 2-way ANOVA with LSD post-hoc analysis). n = 5 – 9 mice per group.


A
ND Veh
DB Veh
ND Ado + ITU
DB Ado + ITU
Mechanical Withdrawal Threshold (g)
0
1
2
3
4
0
0.5
1.5
3
5
Hours post i.t. Ado + ITU
###
####
####
##
****
****
***
*


B
ND Veh
DB Veh
ND CPA
DB CPA
Mechanical Withdrawal Threshold (g)
0
1
2
3
4
0
0.5
1.5
3
5
Hours post i.t. CPA
###
##
***
**

interventions. Identification of the involvement of ectonucleotidases in mitigating pain transmission spurred renewed interest in the effect of diabetes on these enzymes and the downstream activation of important antinociceptive signaling pathways.

Nucleotides, such as ATP, can be released into the extracellular space from vesicles at nerve synapses, and also following tissue injury from damaged and dying cells [67]. These nucleotides can then go on to activate P2X and P2Y receptors (see Chapter 1) expressed throughout the nervous system, resulting in pain sensation and nociceptive neurotransmission [67, 68, 127]. Ectonucleotidases function to terminate this nociceptive signaling by hydrolyzing pro-nociceptive nucleotides into the antinociceptive small molecule adenosine. Recently, Zylka and colleagues demonstrated the important role that PAP and NT5E play in modulating mechanical allodynia and thermal hyperalgesia in rodent models of inflammatory and neuropathic pain [100, 104].

Diabetes affects AMP hydrolysis in the central nervous system

In the DRG, PAP and NT5E have been shown to co-localize extensively with markers that are used to identify nonpeptidergic, putative nociceptive neurons such as P2X3, IB4 and *Mrgprd* [100, 103, 104]. Additionally, both enzymes are expressed in lamina II of the spinal cord where the central projections of these non-peptidergic fibers terminate, placing them in prime location to modulate nociceptive signaling [100, 103, 104]. Immunohistochemistry revealed a similar general pattern of expression for both PAP and NT5E in the DRG and spinal cord of A/J mice. Immunohistochemical analysis of PAP did not reveal any changes in overall expression of PAP between diabetic and nondiabetic mice in the DRG or spinal cord.

Because there was no change in enzyme expression levels, we hypothesized that diabetes may affect enzyme activity, rather than overt expression levels. Our laboratory has previously shown that TMPase activity is decreased in the dorsal horn of diabetic mice, and TMPase was recently identified as the membrane bound form of PAP [97, 100]. NT5E is primarily active near physiological pH (pH 7.0), while PAP is mainly active at acidic pH (pH 5.6); however, both enzymes contribute to AMP hydrolysis *in vivo* [100, 104]. In the current study, the preferred pH of these enzymes was exploited to determine the relative contribution of each enzyme towards AMP hydrolysis. The results revealed decreased hydrolytic activity of AMP following 8 weeks of diabetes at both pH 7.4 and pH 5.6, indicating that the enzymatic activity of both ectonucleotidases was affected by diabetes. At this time, diabetic mice displayed significant mechanical allodynia, suggesting that reduced adenosine production may contribute to their altered sensory phenotype. To our knowledge these are the first studies to show that diabetes has an effect on the hydrolytic capacity of ectonucleotidases in the central nervous system, despite not interfering with protein expression levels.

Endogenous adenosine production can transiently reverse mechanical allodynia

Previous studies have shown that a single i.t. injection of hPAP can alleviate mechanical allodynia and thermal hyperalgesia in rodent models of inflammatory and neuropathic pain for up to three days, and these results were dependent on A_1R activation [100, 110]. Injection of i.t. hPAP was able to improve withdrawal thresholds in diabetic mice compared to baseline after 24 hours, but this effect did not last past 48 hours. This lack of efficacy could be due to chronic hyperglycemia interfering with intrinsic enzyme activity, as was seen using AMP histochemistry,

thus preventing maximal activation of A_1R. Alternatively, it is possible that the peak antinociceptive effect of i.t. hPAP occurred prior to the 24 hour time point in our model of painful diabetic neuropathy, thereby preventing our ability to observe a significant antinociceptive effect following administration of i.t hPAP. Lack of sufficient substrate (AMP) for enzyme activation was ruled out by the observation of significantly increased mechanical withdrawal threshold following i.t. AMP + ITU administration in diabetic mice.

Adenosine is rapidly converted *in vivo* to inosine via adenosine deaminase (ADA) or phosphorylated into AMP by adenosine kinase (AK) [52, 94, 128]. Previous studies have suggested the use of inhibitors for ADA or AK as potential therapeutic treatment options to effectively increase the concentration of adenosine present in the extracellular space [106, 129]. The AK inhibitor, ITU, was co-administered with AMP to promote activation of A_1R. Diabetic mice treated with AMP + ITU displayed a transient reversal of mechanical sensitivity at 30 min following injection, but this returned to pre-treatment withdrawal thresholds within 1.5 hours. This suggests that despite decreased enzymatic activity as a consequence of diabetes, AMP hydrolysis was sufficient to generate enough adenosine to activate A_1R and alter mechanical sensitivity.

Central activation of A_1R modulates nociception in diabetic mice

Several studies have shown that the antinociceptive properties of adenosine are driven by activation of A_1R through the use of selective agonists and antagonists [110, 112, 123, 130-135]. Tian et al. report that A_1R is under tonic activation by adenosine, which may contribute to setting a “physiological nociceptive threshold” [112]. Disturbances in this balance, as suggested

here by decreased enzymatic activity leading to decreased adenosine production, could shift the threshold from a normal pain-free setting to a pro-nociceptive condition that could influence pain states under chronic disease conditions. Additionally, Guieu et al. report that patients with chronic neuropathic pain have lower circulating levels of adenosine in their blood and cerebrospinal fluid, and this loss of tonic activation of antinociceptive pathways could contribute to increased pain sensation in chronic disease settings [136].

As discussed in Chapter 1, A_1R expression has been extensively studied and characterized in the DRG and spinal cord. PAP and NT5E are expressed on a subset of A_1R-expressing neurons, suggesting these enzymes may act locally to modulate nociceptive tone through hydrolysis of AMP and subsequent activation of localized A_1Rs. In this study, i.t. delivery of Ado + ITU was able to significantly reverse mechanical withdrawal thresholds in diabetic mice for over five hours, suggesting that central activation of A_1R may provide an endogenous mechanism for alleviating signs of painful diabetic neuropathy. Because adenosine can act at any of the four adenosine receptors, the stable adenosine analog and direct activator of A_1R, CPA, was used to confirm that the behavioral outcomes observed in response to i.t. Ado + ITU were in fact due to activation of A_1R. This study shows that activation of A_1R by CPA resulted in a reversal in mechanical sensitivity that lasted several hours, similar to adenosine, when compared to indirect activation through hydrolysis of AMP via ectonucleotidases, which was only effective for 30 min following injection. Collectively, these data show that both adenosine and CPA were both capable of alleviating mechanical sensitivity, and these results are likely mediated through activation of A_1R.

Conclusions

In summary, this study utilized a rodent model of painful diabetic neuropathy to evaluate the effect of diabetes on endogenous antinociceptive signaling pathways. For the first time, we show that diabetes significantly decreases the ability of ectonucleotidases, such as PAP and NT5E, on small-diameter, putative nociceptive neurons to hydrolyze AMP leading to decreased production of adenosine. Decreased adenosine production in turn leads to decreased activation of A_1R, loss of inhibition of excitatory interneurons and subsequent increased excitatory signaling. Collectively, loss of tonic activation of A_1R leading to increased excitatory signaling could be one mechanism contributing to the development of painful diabetic neuropathy.

Finally, using a series of *in vivo* experiments we were able to show that central activation of A_1R can successfully alleviate mechanical allodynia associated with painful diabetic neuropathy. Individually, neither AMP, adenosine nor ITU were capable of improving mechanical withdrawal thresholds. Additionally, indirect activation of A_1R following hydrolysis of AMP by either i.t. hPAP or remaining endogenous AMPase activity were only minimally effective at alleviating signs of painful diabetic neuropathy. Conversely, direct activation of A_1R by either adenosine or CPA was extremely effective at reversing mechanical allodynia for several hours. Our results confirm that adenosine-mediated antinociception via A_1R activation can successfully improve mechanical allodynia in a rodent model of painful diabetic neuropathy.

CHAPTER 3

Downstream signaling involved in A_1R-mediated antinociception

1. Abstract

Activation of A_1R is attributed with antinociceptive benefits. Upon activation, the G-protein associated with this GPCR dissociates from the receptor and can go on to activate a variety of second messenger systems, resulting in signal amplification. Pathways downstream of A_1R activation include inhibition of adenylyl cyclase and decreased cAMP production, with subsequent inhibition of PKA activation; Akt activation, presumably through activation of PI3K; inhibition of calcium channel signaling; and modulation of TRPV1 activity. The purpose of this study was to identify pathways are involved in A_1R mediated antinociception. Following i.t. administration of Ado + ITU, there was overall decrease in cAMP levels at 30 min compared to baseline in the spinal cord, and no activation of PKA was detected. Surprisingly, Akt was robustly activated following i.t. administration of the selective A_1R agonist, CPA. Although no changes in calcium channel expression were detected in the spinal cord or DRG of diabetic mice compared to nondiabetic mice, primary DRG cultures from diabetic mice showed an increased area under the curve (AUC) in response to the TRPV1 agonist, capsaicin. Overnight incubation with adenosine was able to return this increased AUC to control levels, as well as decrease the overall percentage of neurons that responded the stimulation with capsaicin. Collectively, these data suggest that cAMP- and Akt-mediated downstream signaling are involved in the antinociceptive actions of A_1R, and that activation of A_1R can modulate calcium homeostasis, in part, through modulation of TRPV1 activity.

2. Introduction

Adenosine signaling in the central nervous system has been implicated in a variety of roles including normal physiological processes as well as in pathological conditions, and has been shown to have neuroprotective benefits when activated under pathological conditions [52, 78]. The outcomes of adenosine-mediated signaling are dependent upon the receptor subtype activated, with a majority of work focused on the downstream signaling effects of A_1R and $A_{2A}R$. These two receptors essentially work in opposition to one another, with activation of $A_{2A}R$ associated with decreased GABAergic signaling and hence increased excitatory signaling, whereas activation of A_1R is associated with decreased excitatory signaling through inhibition of pro-nociceptive neurotransmitter release. Thus, activation of A_1R is attributed with antinociceptive outcomes.

Several second messenger pathways have been associated with adenosine receptor activation which can regulate a variety of intracellular processes. All adenosine receptors are GPCRs and couple primarily to either $G\alpha_s$ or $G\alpha_i$ regulatory α-subunits of the associated heterotrimeric g-protein. Classically, activation of adenosine receptors is linked to modulation of adenylyl cyclase activity and cAMP production [137, 138]. $A_{2A}R$ is coupled to $G\alpha_s$ leading to activation of adenylyl cyclase and increased production of cAMP, while A_1R is coupled to $G\alpha_i$ which inhibits adenylyl cyclase resulting in decreased production of cAMP. Protein kinase A (PKA) activity can be modulated by cAMP levels, and activation of PKA can lead to vesicular release at nerve synapses. For example, cAMP-dependent PKA-mediated signaling in neurons has been linked to neurotransmitter release [139], synaptic plasticity and memory formation

[140], and long-term potentiation [141]. Additionally, PKA can activate cAMP response element binding protein (CREB) which can regulate nuclear gene transcription.

Another important second messenger pathway associated with adenosine receptor activation is that of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB, also known as Akt). Activation of the PI3K/Akt signaling pathway downstream of adenosine receptors has been implicated in a variety of physiological processes, including protection from ischemia-reperfusion-induced renal injury (A_1R-mediated) [142], regulation of vascular smooth muscle tone in the heart (A_{2A}R-mediated) [143], and inhibition of IL-12 production, which can modulate activation of the innate immune system (A_3R-mediated) [144]. Furthermore, in the nervous system, activation of PI3K/Akt signaling downstream of A_1R activation has been shown to have neuroprotective benefits in acute (stroke) and chronic (Alzheimer's disease) neurodegenerative diseases [80], in addition to regulating neuronal excitability through modulation of neurotransmitter release.

Finally, activation of adenosine receptors has been shown to modulate calcium signaling, both intracellularly and extracellularly. Activation of phospholipase C (PLC) downstream of A_1R leads to cleavage of PIP_2 and generation of IP_3 and DAG. Binding of IP_3 to its receptor can mobilize intracellular calcium stores ($[Ca^{2+}]_i$) from the endoplasmic reticulum, which, together with DAG, can activate protein kinase C (PKC) resulting in further propagation of signaling cascades. Additionally, PIP_2 has been shown to modulate the calcium-permeable TRPV1 channel and can be either excitatory or inhibitory depending upon the strength of stimulus used for channel activation (see Chapter 1) [109]. In the context of A_1R activation, it has been proposed that downstream activation of PLC and subsequent cleavage of PIP_2 results in

inhibition of TRPV1 and, therefore, improvement in thermal withdrawal latencies in inflammatory and neuropathic pain models [110].

In Chapter 2 we described how altered adenosine production may contribute to the development of painful diabetic neuropathy. Moreover, we showed that activation of the A_1R-mediated antinociceptive pathway was able to alleviate signs of mechanical allodynia in a rodent model of painful diabetic neuropathy. In the current study, we sought to determine where possible areas of dysregulation are evident, and which downstream pathways were activated following activation of A_1R that might be contributing to the observed antiallodynic effects.

3. Experimental Procedures

Animals

All experiments were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. Inbred male A/J mice were obtained from Jackson Laboratories at 7 weeks of age, and were induced with diabetes at 8 weeks of age (reference Chapter 2). All mice were housed in the Laboratory Animal Resources building, had *ad libitum* access food and water, and maintained on a 12 hour light/dark cycle. All mice were sacrificed at 16 weeks of age.

Western Blot Analysis

At sacrifice, spinal cord and DRG were flash frozen in liquid nitrogen and stored at -80°C until processing. Tissues were sonicated in cell extraction buffer (Invitrogen, Carlsbad, CA) containing 55.5 µl/mL protease inhibitor cocktail (Sigma, St. Louis, MO), 200 mM Na_3VO_4

and 200 mM NaF for 60 min on ice with gentle vortexing every 10 min for protein extraction. Following centrifugation at 7000 rpm for 10 min at 4°C, the protein concentration of the supernatant was determined using the Bradford assay (Bio-Rad, Hercules, CA). Samples were boiled with lane marker reducing sample buffer (Thermo Scientific, Waltham, MA) and stored at -20°C until use. Equal amounts of protein were loaded and separated on a 4-15% gradient Tris-glycine gel (Bio-Rad; 30 mA/gel, 45 min, 4°C), and then transferred to a nitrocellulose membrane (400 mA, 1.5 hours, 4°C). Following incubation with primary and secondary antibodies, bands were visualized using Enhanced Chemiluminesence reagent (ECL; Thermo Scientific). Membranes were exposed to x-ray film and analyzed using Image J software (NIH). All primary antibodies were generated in rabbit unless otherwise noted, and included: anti-A_1R (1:500; Thermo Scientific), anti-TRPV1 (1:5000; Alomone, Jerusulem, Isreal), anti-pAkt (S473) (1:500; Cell Signaling, Danvers, MA), anti-total Akt (1:1000; Cell Signaling), anti-pPKA C-α (1:1000; Cell Signaling), anti-total PKA (1:1000; Cell Signaling), anti-$Ca_v2.2$ (1:250; Origene, Rockville, MD), and mouse anti-α2 (1:1000; Sigma). All secondary antibodies were purchased from Santa Cruz, coupled to horseradish peroxidase and included goat anti-rabbit and donkey anti-mouse.

Membranes were stripped using Restore Plus Western Blot Stripping Buffer (Thermo Scientific), followed by incubation with actin pre-conjugated to HRP (1:10,000; Abcam, Cambridge, MA) overnight at 4°C. Bands were visualized with ECL, membranes exposed to x-ray film and analysis completed using Image J software (NIH).

Determination of cAMP concentration

Prior to sacrifice, nondiabetic and diabetic mice (8 weeks post-STZ; see Chapter 2) were injected with either i.t. Ado + ITU or i.t. CPA. Spinal cord and DRG were harvested at zero (no injection), 30 minutes and 5 hours following injection. Tissues were flash frozen in liquid nitrogen and stored at -80°C until use. A competitive binding ELISA (Enzo Life Sciences) was used to determine intracellular cAMP levels. Samples were analyzed per manufacturer's protocol. Briefly, spinal cord and DRG were sonicated in 0.1 mM HCl (100 µl for spinal cord, 75 µl for DRG). Following centrifugation at 600 x g for 10 min at 4°C, the protein concentration of the supernatant was determined using the Bradford assay (Bio-Rad). Standards were prepared by serial dilution using the supplied standard (2,000 pmol/mL cAMP) and used within one hour of preparation. Samples were diluted (1:20 for spinal cord; 1:10 for DRG) in 0.1mM HCl to fall within range of the standard curve. 100 µl of standards and samples in duplicate were loaded per well with appropriate solutions as indicated. Plates were incubated for 2 hours at room temperature with gentle shaking, washed three times with 400 µl of supplied wash buffer, and then incubated with substrate for one hour at room temperature without shaking. Upon stopping the reaction, the optical density of each plate was determined at 405 nm. Standard curves were generated using Prism software (Graphpad Inc.), and sample concentrations were determined. All samples were normalized to protein concentration. Samples that fell outside of the standard curve were eliminated from analysis.

Determination of mRNA expression levels

RNA was extracted from spinal cord, DRG and skin using TRI Reagent (Sigma) and RNeasy Mini Kit (Qiagen, Valencia, CA). Sample concentration and purity were determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Complimentary DNA (cDNA) was synthesized from 0.5 µg total RNA using the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using SsoFast Probes Supermix Kit (Bio-Rad). The primers used were as follows:

$Ca_v2.2$: Forward: 5'– CCTGATCGTCTACATGCTCTTC – 3'

Reverse: 5' – ATCAAATACTGACCCCTGC – 3'

Ribosomal L27: Forward: 5' – ACAACCACCTCATGCCCACA – 3'

Reverse: 5' – CTGGCCTTGCGCTTCAAA – 3'

All reactions were performed in triplicate. $Ca_v2.2$ mRNA levels (spinal cord and DRG) were normalized to the ribosomal protein rL27. Raw PCR data was analyzed using LinRegPCR software (version 2012.3) to determine PCR efficiency values. Threshold cycle values for L27 were subtracted from the samples, and the percentage of fold change from nondiabetic animals was calculated using the pfaffl method.

Primary DRG cultures

Mice were overdosed with inhaled isoflurane (Pheonix, St. Joseph, MO), injected with 150 µl of heparin (BD Biosciences, San Jose, CA) into the ventricle, and then transcardially perfused with ice-cold Ca^{2+}/Mg^{2+} Hank's Buffered Salt Solution (HBSS; Gibco). Lumbar DRG (L4-L6) were immediately dissected and placed in HBSS for further processing. Dissociation of neurons and culture preparations were prepared as previously described [145]. Briefly, DRG

were partially digested with papain (Worthington, Lakewood, NJ), followed by digestion with collagenase type II (Worthingon) and dispase (Roche, Basel, Switzerland). DRG were triturated through fire-polished glass Pasteur pipettes to dissociate neuronal cell bodies, and then plated on Poly-D-Lysine/Laminin coated glass coverslips (BD Biosciences) placed in 12-well culture plates. Neurons were allowed to adhere to the coverslips for 2-3 hours before incubation in F-12 media (Life Technologies, Grand Island, NY) supplemented with 10% FBS and penicillin/streptomycin (50 U/mL) overnight at 37°C and 5% CO_2 for imaging the next day.

Calcium Imaging

Imaging experiments were performed as previously described [146]. Coverslips were incubated in HBSS with 5 mg/mL BSA (Sigma) and 2 µM fura-2 (Molecular Probes, Invitrogen) for 30 min at 37°C prior to mounting on an inverted Nikon microscope stage. The heated stage was maintained at a constant 30°C, and delivery of HBSS bath solution was controlled by a gravity flow system. Delivery of agonist solutions was accomplished using rapid-switching local perfusion system. Selection of neurons was made using a 480 nm filter and identified as regions of interest using NIS Elements software (Nikon Corporation). To check neuron viability, a brief 5 second application of 30 mM K^+ (high K^+) was applied to the entire field. Neurons were stimulated with capsaicin and SDS following overnight incubation in media alone or media supplemented with 0.1 mM adenosine. Following application of high K^+, neurons were allowed to recover for 5 min, after which 10 µM capsaicin was applied for 10 seconds. Stock capsaicin (10 mM) was prepared in 1-methyl-2-pyrrlidinone; 1 µM capsaicin was made fresh daily for use in experiments. After a 10 min recovery period, neurons were stimulated with 0.006% SDS in HBSS to obtain maximal calcium responses from each individual neuron for normalization

purposes [110]. Absorbance data at 340 and 380 nm were collected at one frame per second. The ratio of 340/380 nm was used to evaluate neuron responses. A peak response of of $\Delta F_{340/380} > 0.1$ was considered positive, and this ratio was used to calculate area under the curve using Excel macros.

Statistical analysis

Results were analyzed using the SPSS Statistics 20 software (IBM). Student's t-tests and two-way analysis of variance (2-way ANOVA) with Fisher's least significant difference (LSD) post-hoc analyses were performed, as denoted in the manuscript. A *p*-value less than 0.05 was considered significant. All data are presented as mean ± S.E.M.

4. Results & Figures

Downstream signaling following A_1R activation by i.t. Ado + ITU or i.t. CPA.

As shown in Chapter 2, diabetic mice showed significant improvement in mechanical withdrawal thresholds following administration of either i.t. Ado + ITU or i.t. CPA. In the current study, several analyses were employed to determine which downstream pathways are activated following these treatment regimens. Activation of A_1R is known to inhibit adenylyl cyclase activity, resulting in decreased production of cAMP and, consequently, decreased activation of PKA. A_1R protein levels were evaluated to determine the effect of diabetes on protein expression. In the spinal cord, the concentration of A_1R protein was trending towards a significant decrease ($p = 0.056$) in diabetic animals at eight weeks post-STZ induction of diabetes (Fig. 14A) compared to nondiabetic mice, however this difference was not considered

significant. No significant differences in the concentration of A_1R protein was seen in the DRG (Fig. 14B) between nondiabetic and diabetic mice.

Competitive binding ELISAs were used to determine intracellular cAMP levels in the spinal cord and DRG of nondiabetic and diabetic mice following i.t. intervention. Following i.t. Ado + ITU, there were no significant differences in cAMP levels between nondiabetic and diabetic mice at any time point (Fig. 15A); however, an overall significant decrease in cAMP levels were seen in the spinal cord after 30 minutes compared to baseline, and this was trending towards significance at 5 hours compared to baseline ($p = 0.064$) (Fig. 15C). In the DRG, no significant differences in cAMP levels were seen between nondiabetic and diabetic mice at any time point (Fig. 15B), and cAMP levels at 30 min were significantly different from baseline (Fig. 15D).

Following administration of i.t. CPA, cAMP levels in the spinal cord at 5 hours post i.t. administration of CPA were significantly elevated compared to baseline in nondiabetic mice (Fig. 16A); no significant differences were seen in cAMP levels in between groups of diabetic mice at any time point. In diabetic mice at 5 hours post-delivery of i.t. CPA, levels of cAMP were significantly decreased compared to nondiabetic mice at the same time point (Fig. 16A). Overall, cAMP levels in the spinal cord following i.t. CPA were not significantly different at any time point (Fig. 16C). In the DRG, cAMP levels were not significantly different between nondiabetic and diabetic mice at any time point (Fig. 16B), nor were there any significant differences in overall cAMP levels (Fig. 16D) at any time point.

Figure 14. Protein levels of A_1R are not affected by diabetes in the spinal cord or DRG. A) Following eight weeks of diabetes, A_1R protein levels in the spinal cord were not significantly different in diabetic (DB) mice compared to nondiabetic (ND) mice ($p > 0.05$ vs. ND; t-test). B) Protein levels of A_1R were not significantly different between ND and DB mice in the DRG ($p > 0.05$ vs. ND; t-test). n = 7 – 9 mice per group.

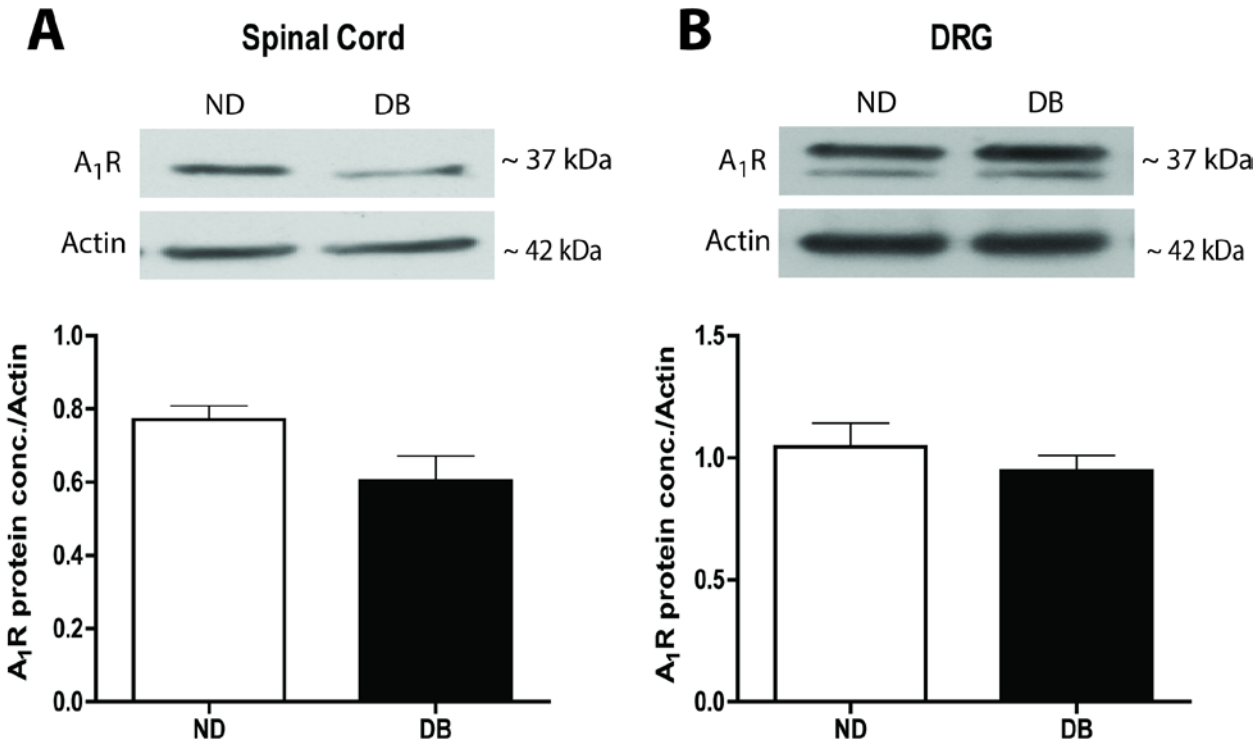

A
Spinal Cord
ND
DB
A1R
~ 37 kDa
Actin
~ 42 kDa
A1R protein conc./Actin
1.0
0.8
0.6
0.4
0.2
0.0
ND
DB
B
DRG
ND
DB
A1R
~ 37 kDa
Actin
~ 42 kDa
A1R protein conc./Actin
1.5
1.0
0.5
0.0
ND
DB

Figure 15. cAMP production in the spinal cord and DRG following i.t. Ado + ITU. A, C) No significant differences in cAMP levels were seen between nondiabetic and diabetic mice at any time point (n = 4 – 6 mice per group), but an overall significant decrease in cAMP levels were seen in the spinal cord at 30 min following i.t. Ado + ITU compared to baseline (# $p < 0.05$ vs baseline; 2-way ANOVA with LSD post-hoc analysis). n = 9 – 10 mice per group. B, D) In the DRG, no differences in cAMP levels were detected between nondiabetic and diabetic mice at any time point ($p > 0.05$ vs. baseline; 2-way ANOVA with LSD post-hoc analysis). n = 4 – 6 mice per group. Overall, cAMP levels in the DRG were not significantly decreased at 30 min or 5 hours compared to baseline following i.t. administration of Ado + ITU ($p > 0.05$ vs. baseline; 2-way ANOVA with LSD post-hoc analysis). n = 8 – 10 mice per group.

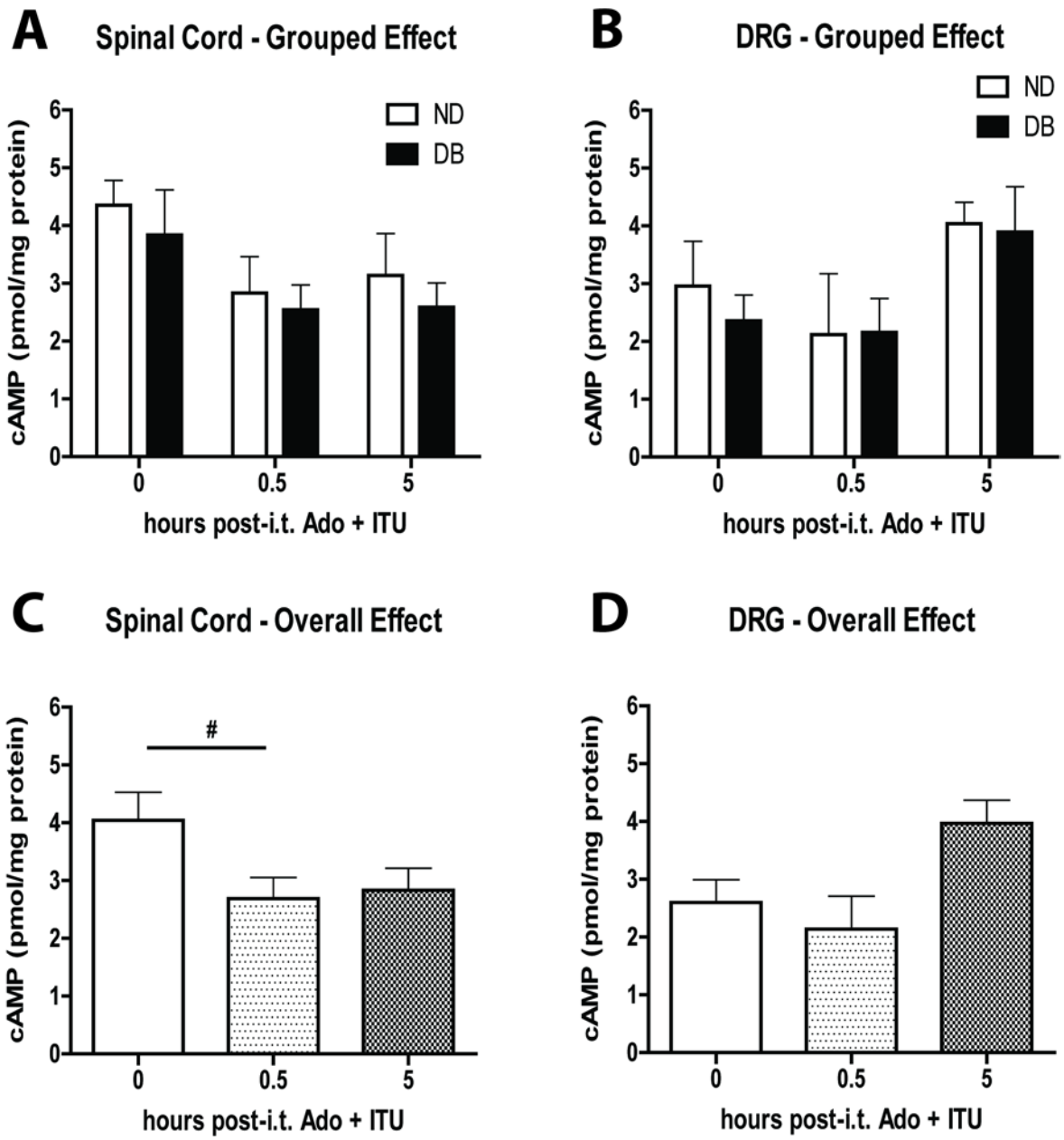
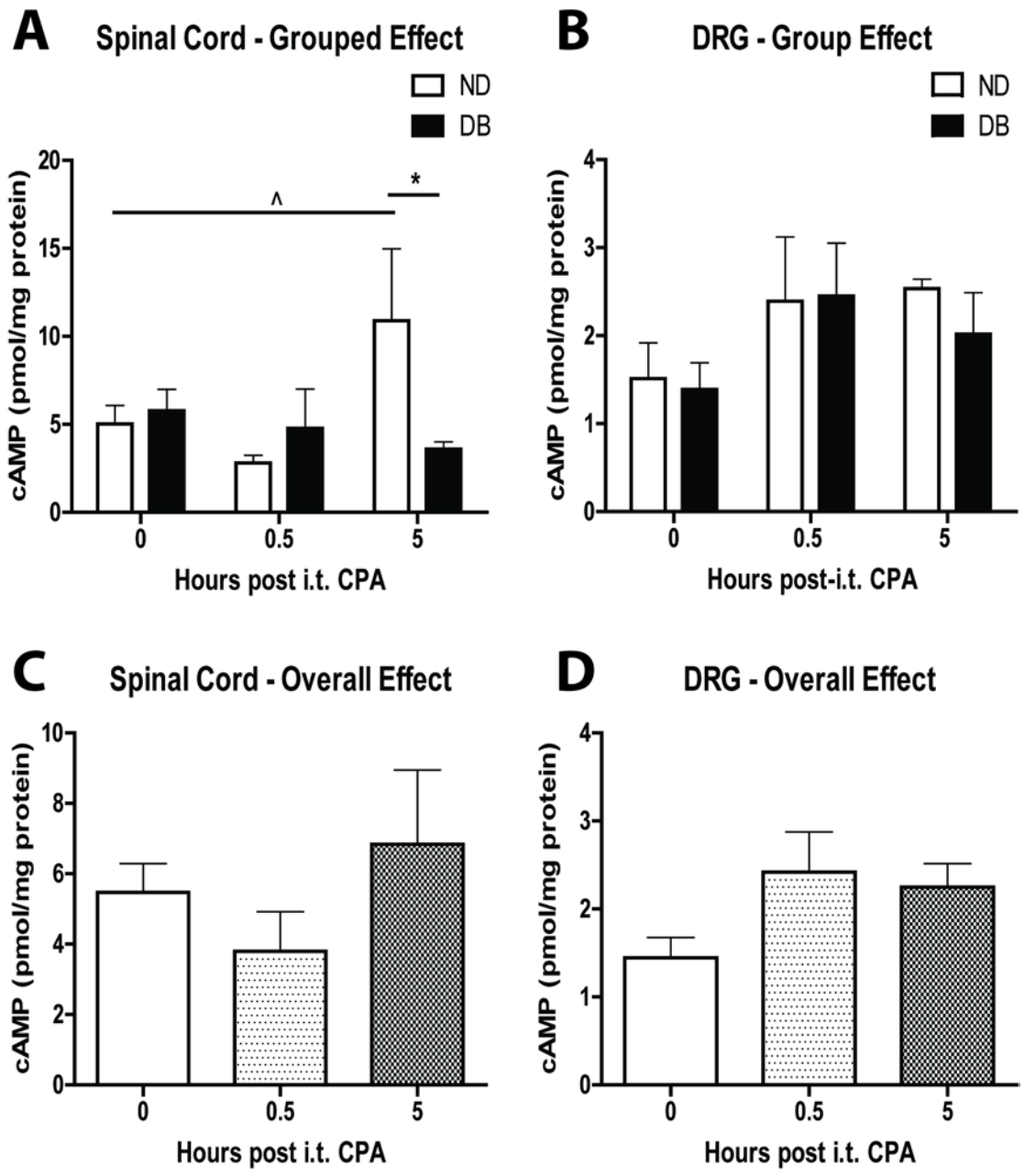

A
Spinal Cord - Grouped Effect
ND
DB
cAMP (pmol/mg protein)
0
1
2
3
4
5
6
0
0.5
5
hours post-i.t. Ado + ITU
B
DRG - Grouped Effect
ND
DB
cAMP (pmol/mg protein)
hours post-i.t. Ado + ITU
C
Spinal Cord - Overall Effect
#
cAMP (pmol/mg protein)
hours post-i.t. Ado + ITU
D
DRG - Overall Effect
cAMP (pmol/mg protein)
hours post-i.t. Ado + ITU

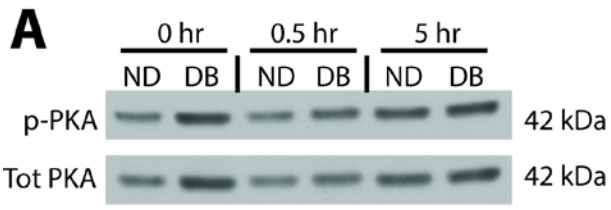
Figure 16. cAMP production in the spinal cord and DRG following i.t. CPA. A, C) cAMP levels in nondiabetic (ND) mice were significantly elevated at 5 hours compared to cAMP levels at 5 baseline (^ $p < 0.05$ vs. baseline; 2-way ANOVA with LSD post-hoc analysis). n = 4 – 6 mice per group. Overall cAMP levels were not significantly different at any time point in the spinal cord ($p > 0.05$ vs. baseline; 2-way ANOVA with LSD post-hoc analysis). n = 9 – 10 mice per group. B, D) In the DRG, no differences in cAMP levels were detected between nondiabetic and diabetic mice at any time point (n = 4 – 5 mice per group), nor were there any significant differences in overall cAMP levels ($p > 0.05$ vs. baseline; 2-way ANOVA with LSD post-hoc analysis). n = 9 mice per group.

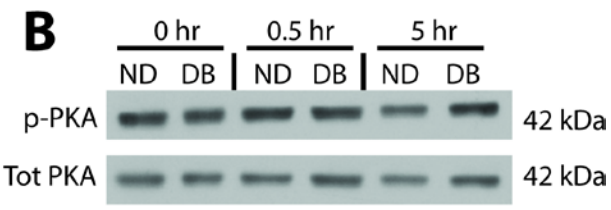

A
Spinal Cord - Grouped Effect
ND
DB
cAMP (pmol/mg protein)
20
15
10
5
0
^
*
0
0.5
5
Hours post i.t. CPA
B
DRG - Group Effect
ND
DB
cAMP (pmol/mg protein)
4
3
2
1
0
0
0.5
5
Hours post-i.t. CPA
C
Spinal Cord - Overall Effect
cAMP (pmol/mg protein)
10
8
6
4
2
0
0
0.5
5
Hours post i.t. CPA
D
DRG - Overall Effect
cAMP (pmol/mg protein)
4
3
2
1
0
0
0.5
5
Hours post i.t. CPA

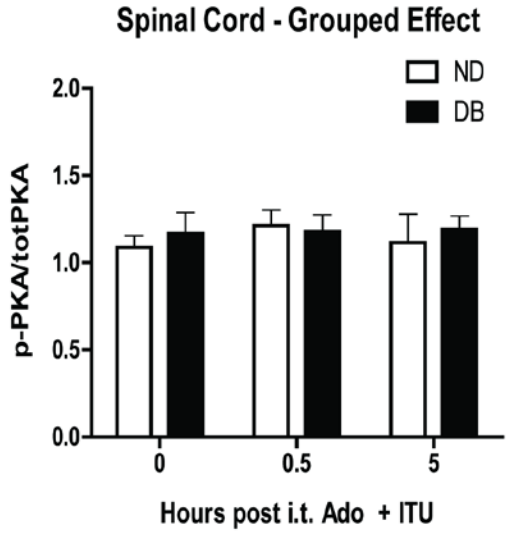
Many downstream events dependent on cAMP signaling are mediated through activation of PKA [137, 147]. Here, activation of PKA was evaluated in the spinal cord following i.t. administration of Ado + ITU or CPA. Representative blots are shown following delivery of i.t. Ado + ITU (Fig. 17A) or i.t CPA (Fig. 17B). Activation of PKA was determined by comparing phosphorylated PKA-Cα (p-PKA) to total PKA. Activation of PKA was not detected in the spinal cord following i.t. Ado + ITU (Fig. 17A) or i.t. CPA (Fig. 17A) between nondiabetic and diabetic mice at any time point, nor were there any differences in overall PKA activation following either treatment (Fig. 17C, D).

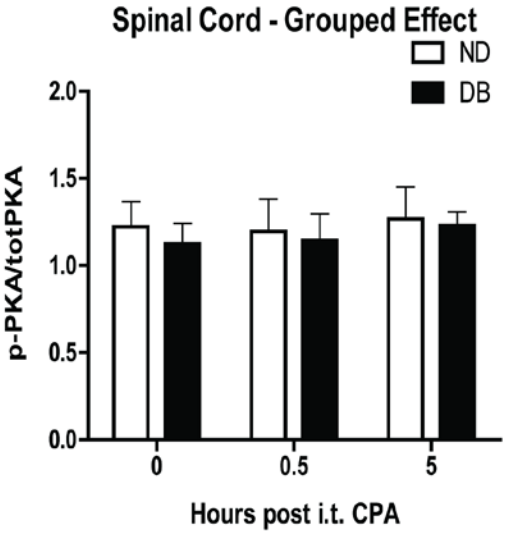
Activation of A_1R has been associated with downstream activation of PI3K and Akt [148, 149]. Activation of Akt in the spinal cord was evaluated following administration of i.t. Ado + ITU and i.t. CPA. Representative blots are shown following i.t. administration of Ado + ITU (Fig. 18A) or CPA (Fig. 18B). Following delivery of i.t. Ado + ITU, no significant changes in the activation of Akt were detected between nondiabetic and diabetic mice (Fig. 18A), nor were any significant changes seen in overall Akt activation (Fig. 18C), at any time point. Interestingly, Akt activation in diabetic mice does appear to be elevated over nondiabetic mice at all time points, although this increase is not significantly different between nondiabetic and diabetic mice. Following administration of i.t. CPA, Akt activation at 30 min was significantly elevated compared to baseline within nondiabetic mice and within diabetic mice (Fig. 18B). Additionally, Akt activation in diabetic mice was significantly elevated at 5 hours compared to nondiabetic mice at this same time point. Furthermore, overall Akt activation at 30 min was significantly elevated compared to baseline (Fig. 18D).

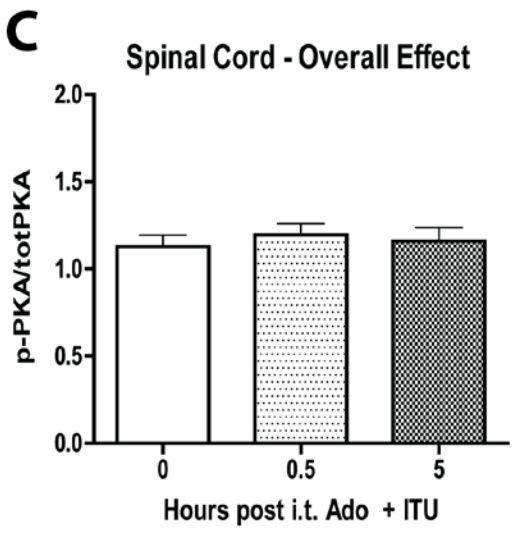
Figure 17. Administration of i.t. Ado + ITU or i.t. CPA does not affect activation of PKA. Representative blots are shown for PKA activation in the spinal cord following i.t. Ado + ITU (A) and i.t. CPA (B). No significant changes in the activation of PKA following i.t. delivery of Ado + ITU (A, C) or i.t. CPA (B, D) were detected between nondiabetic (ND) and diabetic mice (A, B; n = 4 – 8 mice per group), nor were there any overall changes in activation of PKA (C, D; n = 9 – 16 mice per group) at any time point ($p > 0.05$ vs. ND; 2-way ANOVA with LSD post-hoc analysis).


A
0 hr
0.5 hr
5 hr
ND DB ND DB ND DB
p-PKA
Tot PKA
42 kDa
42 kDa


B
0 hr
0.5 hr
5 hr
ND DB ND DB ND DB
p-PKA
Tot PKA
42 kDa
42 kDa


Spinal Cord - Grouped Effect
ND
DB
p-PKA/totPKA
2.0
1.5
1.0
0.5
0.0
0
0.5
5
Hours post i.t. Ado + ITU


Spinal Cord - Grouped Effect
ND
DB
p-PKA/totPKA
2.0
1.5
1.0
0.5
0.0
0
0.5
5
Hours post i.t. CPA


C
Spinal Cord - Overall Effect
p-PKA/totPKA
2.0
1.5
1.0
0.5
0.0
0
0.5
5
Hours post i.t. Ado + ITU

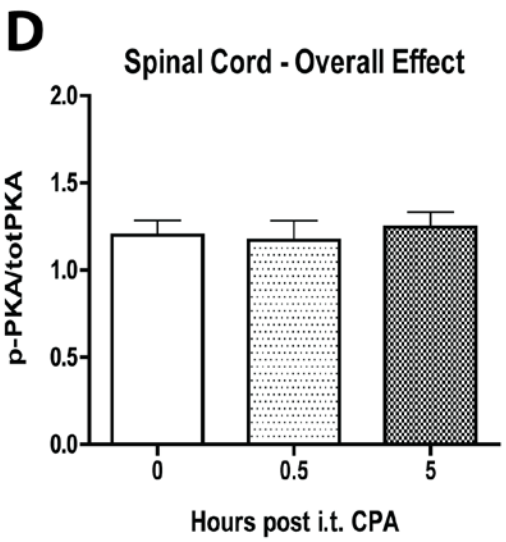
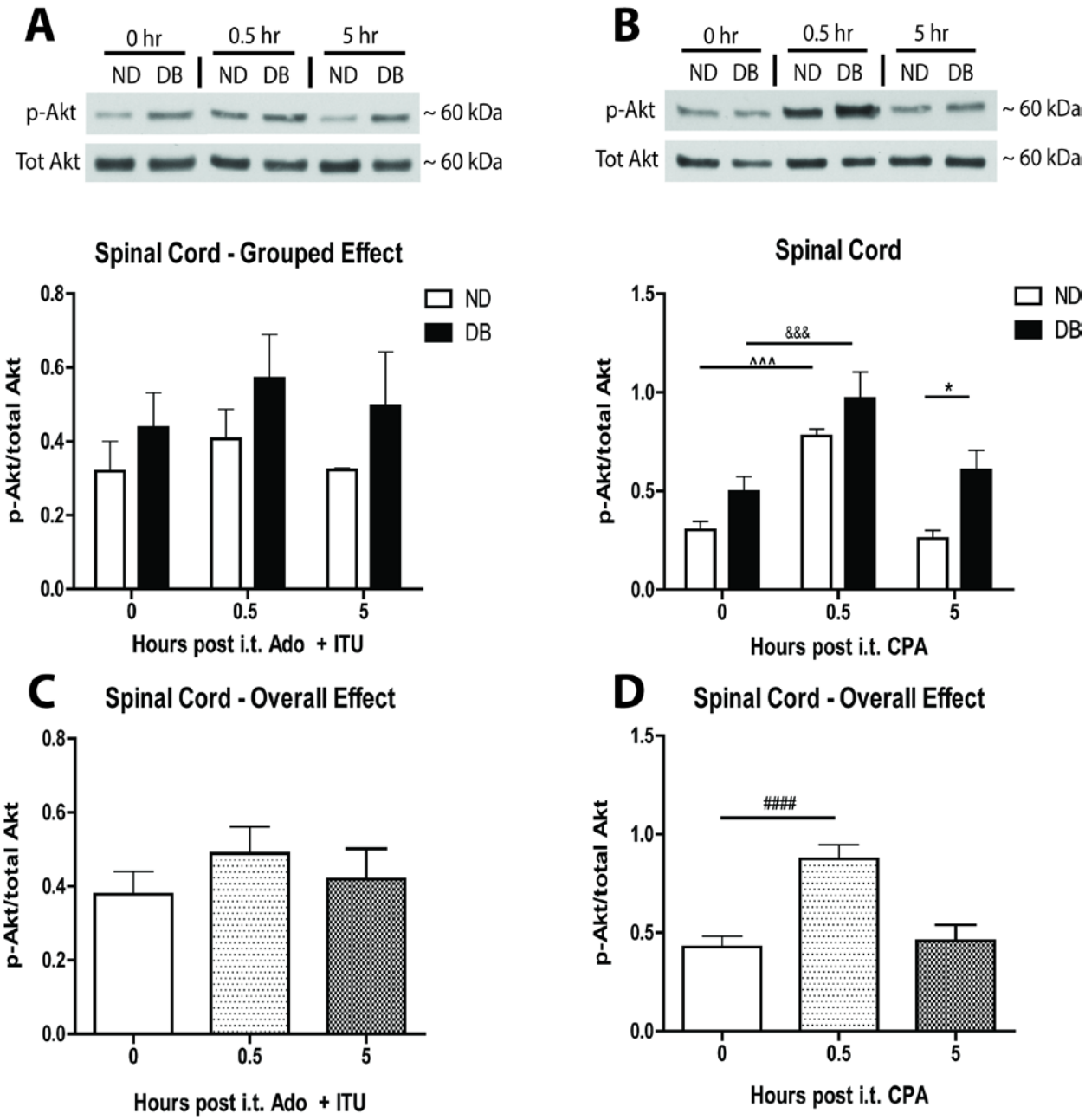

D
Spinal Cord - Overall Effect
p-PKA/totPKA
2.0
1.5
1.0
0.5
0.0
0
0.5
5
Hours post i.t. CPA

Figure 18. Akt activation in the spinal cord is elevated in diabetic mice following administration of i.t Ado + ITU and i.t. CPA. A) Activation of Akt in the spinal cord of diabetic (DB) mice was not significantly different compared to nondiabetic (ND) mice at any time point following administration of i.t. Ado + ITU ($p > 0.05$ vs. ND; 2-way ANOVA with LSD post-hoc analysis). n = 4 – 8 mice per group. C) Overall, activation of Akt was not significantly different at any time point ($p > 0.05$, 2-way ANOVA with LSD post-hoc analysis). n = 9 – 16 mice per group. B) Following administration of i.t. CPA, activation of Akt was significantly elevated at 30 min compared to baseline in both ND mice (^^^ $p < 0.001$ vs. ND; 2-way ANOVA with LSD post-hoc analysis) and DB mice ($^{\&\&\&}$ $p < 0.001$ vs. DB; 2-way ANOVA with LSD post-hoc analysis). Additionally, activation of Akt was significantly elevated in DB mice compared to ND mice at 5 hours (* $p < 0.05$ vs. ND 5 hours; 2-way ANOVA with LSD post-hoc analysis). n = 4 – 6 mice per group. D) Overall, activation of Akt at 30 min was significantly elevated compared to baseline (#### $p < 0.0001$ vs baseline; 2-way ANOVA with LSD post-hoc analysis). n = 9 – 10 mice per group.


A
0 hr
0.5 hr
5 hr
ND DB ND DB ND DB
p-Akt
Tot Akt
~ 60 kDa
~ 60 kDa
B
0 hr
0.5 hr
5 hr
ND DB ND DB ND DB
p-Akt
Tot Akt
~ 60 kDa
~ 60 kDa
Spinal Cord - Grouped Effect
ND
DB
p-Akt/total Akt
0.0
0.2
0.4
0.6
0.8
0
0.5
5
Hours post i.t. Ado + ITU
Spinal Cord
ND
DB
&&&
^^^
*
p-Akt/total Akt
0.0
0.5
1.0
1.5
0
0.5
5
Hours post i.t. CPA
C
Spinal Cord - Overall Effect
p-Akt/total Akt
0.0
0.2
0.4
0.6
0.8
0
0.5
5
Hours post i.t. Ado + ITU
D
Spinal Cord - Overall Effect
####
p-Akt/total Akt
0.0
0.5
1.0
1.5
0
0.5
5
Hours post i.t. CPA

Calcium channel expression and signaling.

Dysregulation of calcium channel expression and signaling has been associated with diabetic neuropathy and nerve injury [51, 150]. Specifically, the $\alpha_2\delta$-subunit of calcium channels has been shown to be upregulated in the spinal cord and DRG following nerve injury [151]. Expression of the $\alpha_2\delta$-subunit in the spinal cord and DRG was determined using western blot analysis. No change in α_2-subunit protein levels were detected in the spinal cord (Fig. 19A) or DRG (Fig. 19B) between nondiabetic and diabetic mice. Activation of A_1R is known to inhibit N-type calcium channels ($Ca_v2.2$) [77]. Therefore, $Ca_v2.2$ mRNA expression in the spinal cord and DRG were evaluated using qRT-PCR. Relative expression of $Ca_v2.2$ mRNA levels were not significantly different in diabetic mice compared to nondiabetic mice in the spinal cord (Fig. 19C) or DRG (Fig. 19D).

Calcium flux through TRPV1 is increased in diabetic mice.

Activation of A_1R results in activation of PLC which can then cleave PIP_2, and PIP_2 has been shown to modulate TRPV1 activity, thus linking activation of A_1R to modulation of TRPV1 signaling. Additionally, changes in TRPV1 expression and signaling have been implicated in diabetes-induced thermal changes [152-154]. TRPV1 protein levels were determined using western blot analysis in the spinal cord and DRG following eight weeks of STZ-induced diabetes. No significant differences in protein levels of TRPV1 were detected between diabetic and nondiabetic mice in the spinal cord (Fig. 20A) or DRG (Fig. 20B).

Figure 19. Diabetes does not affect calcium channel expression in the spinal cord or DRG. No significant changes in α2-subunit protein levels were detected using western blot analysis in the spinal cord (A; n = 8 mice per group) or DRG (B; n = 7 mice per group) of nondiabetic (ND) and diabetic (DB) mice ($p > 0.05$ vs. ND; t-test). Additionally, $Ca_v2.2$ mRNA concentration was not significantly different between ND and DB mice in the spinal cord (C) or DRG (D) ($p > 0.05$ vs. ND; t-test). n = 6 mice per group.

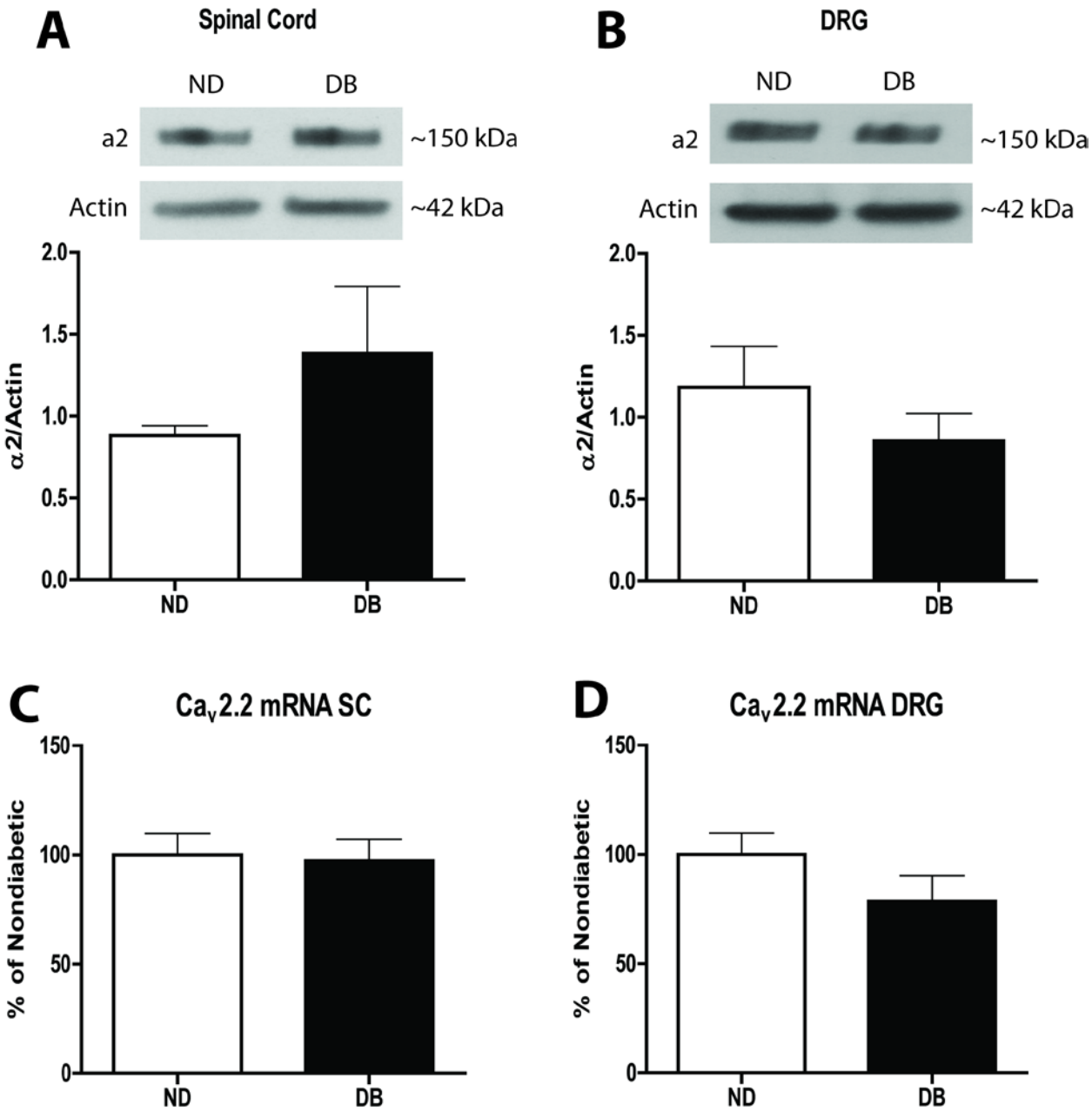
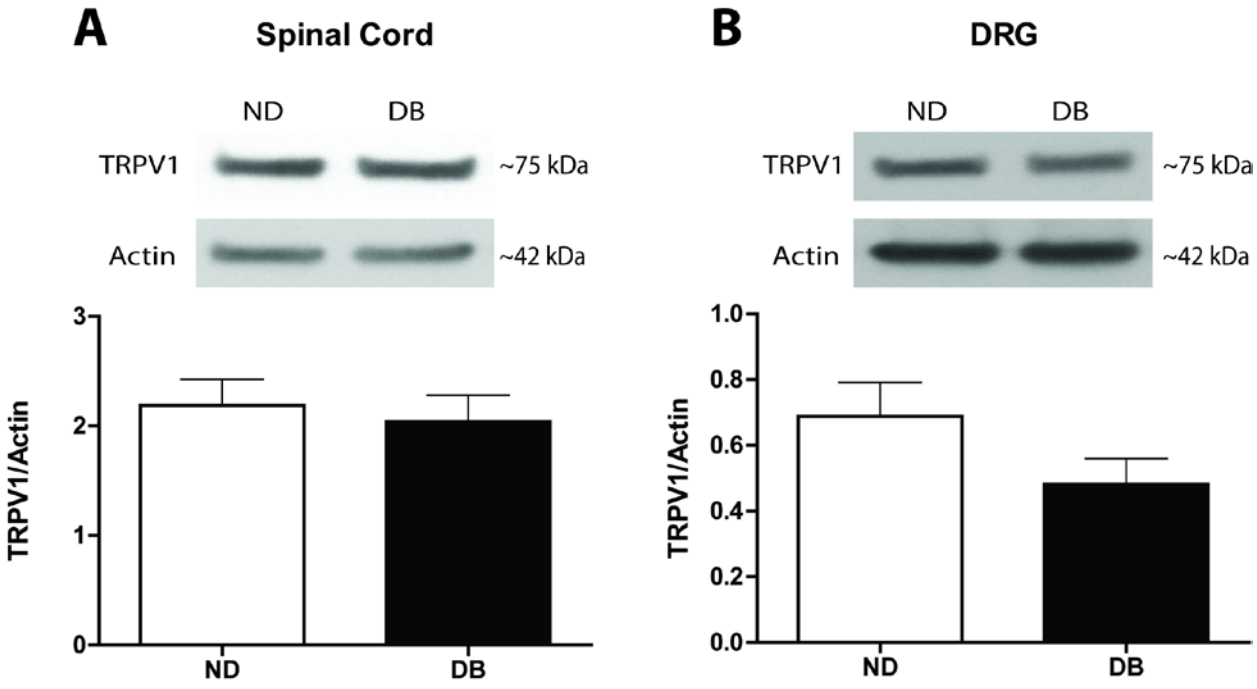

A
Spinal Cord
ND
DB
a2
~150 kDa
Actin
~42 kDa
α2/Actin
2.0
1.5
1.0
0.5
0.0
ND
DB
B
DRG
ND
DB
a2
~150 kDa
Actin
~42 kDa
α2/Actin
2.0
1.5
1.0
0.5
0.0
ND
DB
C
Cav2.2 mRNA SC
% of Nondiabetic
150
100
50
0
ND
DB
D
Cav2.2 mRNA DRG
% of Nondiabetic
150
100
50
0
ND
DB

Figure 20. Protein levels of TRPV1 are not affected by diabetes in the spinal cord or DRG. A) Protein levels of TRPV1 in the spinal cord were not different between nondiabetic (ND) and diabetic (DB) mice ($p > 0.05$ vs. ND; t-test). n = 8 – 9 mice per group. B) In the DRG, protein levels of TRPV1 were not significantly different in DB mice compared to ND controls ($p > 0.05$ vs. ND; t-test). n = 7 – 8 mice per group.

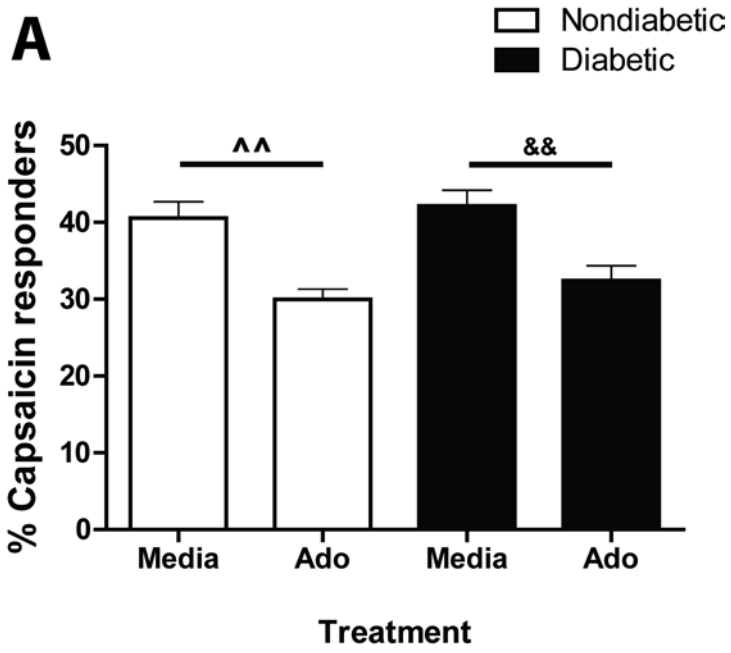

A
Spinal Cord
ND
DB
TRPV1
~75 kDa
Actin
~42 kDa
TRPV1/Actin
3
2
1
0
ND
DB
B
DRG
ND
DB
TRPV1
~75 kDa
Actin
~42 kDa
TRPV1/Actin
1.0
0.8
0.6
0.4
0.2
0.0
ND
DB

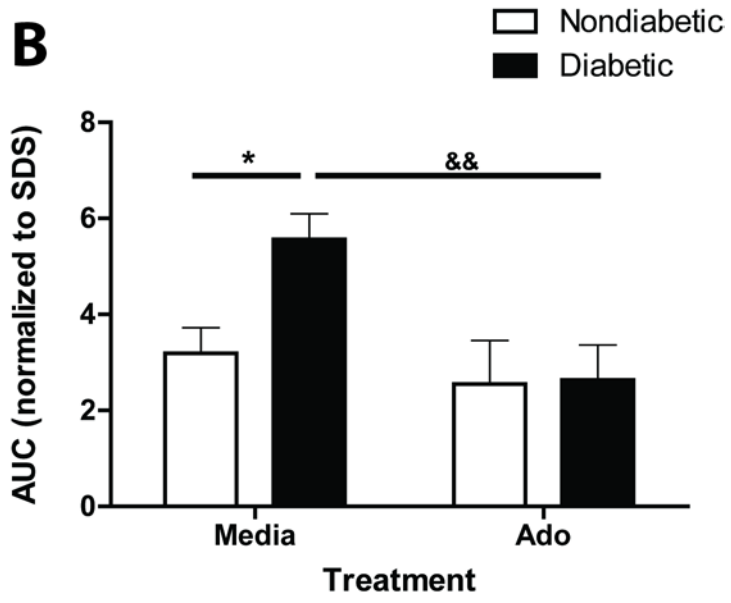
Sowa et al. proposed that activation of A_1R modulates TRPV1 activity through hydrolysis of PIP_2 [110]. Thus, primary DRG cultures from nondiabetic and diabetic mice were incubated overnight with or without adenosine and then stimulated with capsaicin to determine if adenosine-mediated activation of A_1R modulated capsaicin-induced calcium flux through TRPV1. Overnight incubation with adenosine significantly decreased the percentage of neurons that responded to capsaicin-mediated activation of TRPV1 (Fig. 21A). Area under the curve (AUC) analysis revealed that primary DRG cultures from diabetic mice had a significantly increased AUC compared to nondiabetic mice in the absence of adenosine (Fig. 21B). Furthermore, the AUC from primary DRG cultures from diabetic mice incubated overnight with adenosine was significantly decreased compared to the AUC from primary DRG cultures from diabetic media-only treated cultures, and this decreased AUC was not significantly different from control, nondiabetic media- and adenosine-treated cultures.

5. Discussion

In light of the behavioral outcomes discussed in Chapter 2, this study sought to determine what pathways were activated downstream of A_1R that could influence mechanical sensitivity in painful diabetic neuropathy. To accomplish this task, a variety of molecular techniques were employed to investigate several of the second messenger pathways associated with activation of A_1R, including cAMP production, activation of PKA, activation of Akt, and calcium channel expression and signaling. Levels of cAMP were not significantly different between diabetic and

Figure 8. Calcium flux through TRPV1 in primary DRG cultures is increased in diabetic mice, and is restored by overnight incubation adenosine. A) In the presence of adenosine, the overall percentage of neurons that responded to stimulation with capsaicin was significantly decreased in both nondiabetic (ND) mice (^^ $p < 0.01$ vs. ND Media; 2-way ANOVA with LSD post-hoc analysis), and diabetic (DB) mice ($^{\&\&}$ $p < 0.01$ vs. DB Media; 2-way ANOVA with LSD post-hoc analysis) relative to cultures incubated with media only. B) Area under the curve (AUC) analysis revealed that DB mice had a significantly increased AUC in response to stimulation with capsaicin compared to ND controls (* $p < 0.05$ vs. ND Media; 2-way ANOVA with LSD post-hoc analysis). Overnight incubation with adenosine (Ado) reversed this increased AUC to ND control levels ($^{\&\&}$ $p < 0.01$ vs. DB Media; 2-way ANOVA with LSD post-hoc analysis).


A
Nondiabetic
Diabetic
% Capsaicin responders
50
40
30
20
10
0
^^
&&
Media
Ado
Media
Ado
Treatment


B
Nondiabetic
Diabetic
AUC (normalized to SDS)
8
6
4
2
0
*
&&
Media
Ado
Treatment

nondiabetic mice; however, when groups were combined a significant decrease in cAMP levels in the spinal cord was observed following i.t. administration of Ado + ITU. At this same time, no activation of PKA was detected. Interestingly, Akt levels in diabetic mice were elevated compared to nondiabetic mice at all time points following treatment with i.t. Ado + ITU, although these differences were not statistically significant. Additionally, at 30 min following i.t. administration of CPA, a robust activation of Akt was observed in the spinal cord within nondiabetic mice and within diabetic mice. A significant increase in the activation of Akt in diabetic mice compared to nondiabetic mice was evident at 5 hours following i.t. delivery of CPA. Furthermore, these studies show that calcium signaling through TRPV1 is dysregulated in our model of painful diabetic neuropathy, and that treatment of primary DRG cultures overnight with adenosine is able to restore increased calcium flux through TRPV1 in diabetic mice to levels comparable to nondiabetic mice.

cAMP production is decreased following central delivery of A_1R agonists

Classically, A_1R activation is linked to inhibition of andenylyl cyclase through activation of $G\alpha_i$, leading to decreased production of cAMP levels [138]. Results of studies shown here do not reveal any differences in cAMP levels between nondiabetic and diabetic mice, suggesting that the adenosine receptor itself is still able to signal properly in diabetic mice. However, when groups are combined, an overall decrease in cAMP levels at 30 min following i.t. administration of Ado + ITU compared to baseline was detected, suggesting that improvement in mechanical allodynia subsequent to A_1R activation is mediated through inhibition of cAMP production and likely involves activation of A_1R. Additionally, A_1R protein levels in the spinal cord of diabetic

mice were trending towards a significant decrease. Taken together, these data suggest that it is altered receptor expression and activation, and not receptor function, which may underlie the development of painful diabetic neuropathy.

PKA is activated following increases in cAMP levels [155], which can phosphorylate and activate CREB. CREB activation regulates the expression of a number of genes, including *c-fos* and *c-jun*, both of which have been implicated in neuropathic pain conditions [156, 157]. Inhibition of CREB through i.t. injection of antisense oligonucleotides has been shown to alleviate mechanical allodynia in rats following partial sciatic nerve ligation [158]. The data presented here suggests that inhibition of cAMP production in the spinal cord, and therefore inhibition of activation of PKA and subsequent phosphorylation of CREB might prevent expression of *c-fos* and *c-jun*, providing a mechanism by which activation of A_1R may contribute to alleviating mechanical allodynia in this rodent model of painful diabetic neuropathy. Future studies should explore CREB phosphorylation, as well as *c-fos* and *c-jun* expression in nondiabetic and diabetic mice, and following i.t. Ado + ITU treatment to evaluate the involvement of this signaling pathway and downstream mediators in A_1R-mediated antinociception in painful diabetic neuropathy.

Following i.t. CPA, no significant differences in cAMP levels were detected at either 30 min or 5 hours compared to baseline. However, when looking at group differences it should be noted that the significant differences seen between the time points in nondiabetic mice are likely a consequence of the method of analysis rather than actual physiological differences. There were many steps in the analysis that could contribute to this variability, such as overall protein concentration determination (each sample was normalized to individual protein concentration),

variability between duplicate samples on the plate, pipetting errors (samples were diluted to fall within range of the standard curve, pipetting of sample during plate loading), and small sample number. Sample sizes for all cAMP analyses were small (n = 4-6 per group, per time point, per treatment paradigm), and increases in sample number would likely eliminate these differences, as well as strengthen the differences observed in the Ado + ITU treated mice. Additionally, these time points were chosen for analysis because they correlate with observed behavioral outcomes. Future work should consider earlier time points (between baseline and 30 min) to capture early, rapid changes in cAMP levels [155].

Activation of Akt contributes to the antinociceptive actions of A_1R activation

Akt is a key intracellular second messenger involved in a variety of cellular processes, such as cell growth, proliferation, metabolism, and it is activation of this signaling pathway that is attributed with a majority of the metabolic actions of insulin [159]. Phosphorylation of Akt is upregulated following activation of the insulin receptor, and Grote et al. recently reported blunted Akt signaling in the peripheral nervous system of *ob/ob* mice, suggesting that altered Akt signaling may contribute to the development of peripheral neuropathy [160]. Surprisingly, following i.t. delivery of CPA, significantly elevated levels of activated Akt were detected in nondiabetic mice and in diabetic mice, and this increased activation was significantly different at the 5 hour time point between nondiabetic and diabetic mice. Additionally, overall Akt activation was significantly elevated at 30 min compared to baseline following administration of i.t. CPA, suggesting that Akt signaling may be involved in mediating the antinociceptive benefits of activation of A_1R.

Several studies have reported the role of Akt signaling in the maintenance of pain conditions, including recruitment of sodium channels in the development of CFA-induced inflammatory pain [161], opioid-induced tolerance and hyperalgesia in the spinal cord [162], and visceral pain conditions [163]. Conversely, others have reported that activation of PI3K/Akt signaling is involved in morphine-induced peripheral analgesia mediated by kappa opioid receptors [164, 165], as well as relief from neuropathic pain following treatment with the anti-hypercholesterolemic drug atorvastatin [166], and that activation of PI3K/Akt provides a neuroprotective benefit in cortical neurons [149]. The data presented here support the later hypothesis, such that activation of Akt is involved in mediating the antinociceptive effects of i.t. Ado +ITU and i.t. CPA, providing new insight on the contribution of activation of Akt in A_1R-mediated antinociception.

Voltage-gated calcium channels are a diverse group of channels involved in many aspects of cell signaling. Activation of A_1R has been shown to inhibit P-, Q- and N-type calcium channels, and dysregulation of N-type calcium channels has been reported in diabetic rats [167, 168]. Additionally, the $\alpha_2\delta$-subunit of calcium channels has been reported to be upregulated following nerve injury, specifically in the DRG and in presynaptic nerve terminals [169, 170]. Dolphin et al. report that deletion of the $\alpha_2\delta$-subunit gene delays development of mechanical sensitivity [171]. No significant changes in α_2-protein levels in either the spinal cord or DRG were detected in diabetic mice compared to nondiabetic mice, nor were there any changes in $Ca_v2.2$ mRNA expression levels in the spinal cord or DRG between groups. However, there have been reports that $Ca_v2.2$ mRNA levels are actually down-regulated following sciatic nerve ligation [172]. Additionally, the experiments performed in this study were carried out at a

terminal time point, at eight weeks following STZ-induction of diabetes, when diabetic mice have reached a minimum threshold in terms of their mechanical sensitivity. There is likely temporal regulation of calcium gene and protein expression and the terminal time points at which these experiments were performed were beyond the window in which to capture changes in relative expression. Future experiments should address the effect of time on differential regulation and expression of calcium channels.

Diabetic male A/J mice do not develop changes in thermal sensitivity (unpublished data) so it is not surprising that there were no changes in TRPV1 protein expression levels between nondiabetic and diabetic mice. Interestingly, it appears that TRPV1 may be slightly decreased in the DRG of diabetic mice, however, this difference was not statistically significant. Indeed, Hong and Wiley report that TRPV1 expression is decreased in the DRG of diabetic rats when using whole cell homegnates for western blot analysis, but was enriched in the plasma membrane [154]. Despite the lack of thermal hyperalgesia evident in the current model of painful diabetic neuropathy, alterations in calcium flux through the TRPV1 channel following activation with capsaicin were significantly increased in primary DRG cultures from diabetic mice. This suggests that altered TRPV1 signaling contributes to dysregluated calcium homeostasis in this model system and may contribute to the development of painful neuropathy. Furthermore, overnight treatment of primary DRG cultures from diabetic mice with adenosine blunts this altered signaling, strengthening the argument that activation of A_1R inhibits TRPV1 activity, presumably through PLC-mediated cleavage of PIP_2, providing a mechanism by which calcium signaling can be modulated *in vivo*.

Conclusions

Several downstream signaling pathways are associated with activation of A_1R. The experiments performed in this chapter were done in an attempt to identify those pathways which may be involved in mediating the antinociceptive behavioral outcomes observed in Chapter 2 following i.t. administration of Ado + ITU and i.t. CPA. No changes in cAMP levels were detected between diabetic and nondiabetic mice following either i.t. Ado + ITU or i.t. CPA, suggesting that diabetes does not affect the ability of A_1R to signal properly. Additionally, activation of PKA was not observed, suggesting that inhibition of cAMP-mediated activation of PKA was prevented following these pharmacological interventions, and may suggest a mechanism by which activation of A_1R contributes to alleviating mechanical sensitivity in painful diabetic neuropathy. Surprisingly, a robust activation of Akt in the spinal cord was observed following i.t. administration of CPA, and this increased activation was significantly different between nondiabetic and diabetic mice at 5 hours following delivery of CPA. This suggests that the PI3K/Akt second messenger pathway may be involved in A_1R-mediated antinociception in painful diabetic neuropathy. Finally, increased calcium flux through neurons in primary DRG cultures from diabetic mice in response to the TRPV1 agonist capsaicin was observed, and this heightened response was blunted by overnight incubation with adenosine, suggesting that adenosine can regulate calcium homeostasis and the mechanism by which the occurs by be through activation of A_1R.

CHAPTER 4

Peripheral delivery of specific A_1R agonist reveals a novel mechanism for the treatment of painful diabetic neuropathy

1. Abstract

Current strategies for the treatment of painful diabetic neuropathy primarily rely on the use of oral medications. However, these agents come with varying efficacy between individuals and carry significant risk of systemic adverse effects. Thus, there has been a recent impetus to move away from systemic (oral) treatment regimens and move towards peripheral routes of delivery (topical treatment) that minimize risk of systemic adverse effects. In this chapter, we show that A_1R is expressed in the hind paw skin of nondiabetic and diabetic mice, and that diabetes does not affect the protein levels of this receptor. Additionally, we show that peripheral (intraplantar) delivery of a specific and selective A_1R agonist, CPA, significantly reverses mechanical allodynia in diabetic mice. This shows that peripheral administration of A_1R-specific agonists is effective at alleviating signs of mechanical allodynia associated with painful diabetic neuropathy, and that further development of peripheral routes of delivery can provide a novel therapeutic treatment option for individuals suffering from painful diabetic neuropathy.

2. Introduction

Current treatment options for painful diabetic neuropathy are limited to symptomatic treatment, have variable efficacy, and come with significant risk of side effects [9, 17, 114]. Therapies currently in use for the management of painful diabetic neuropathy include antidepressants (ex: tricyclic antidepressants and selective serotonin-norepinephrine reuptake inhibitors), opioids (ex: oxycodone, tramadol), antiepileptic drugs (ex: gabapentin, pregabalin), and topical agents (ex: capsaicin cream, lidocaine cream). Notably, none of these treatment options are aimed at the pathogenetic mechanisms thought to underlie the development of painful diabetic neuropathy. To date, the only proven disease modifying intervention effective in delaying the progression of diabetic neuropathy is tight glycemic control [9, 10, 27, 28].

Several pathogenetic treatment options are under investigation, but none are licensed for use in the United States and few have made it successfully through clinical trials [9, 33, 117]. One such treatment option, Epalrestat, an aldose reductase inhibitor, is currently licensed for use in Japan and India. Epalrestat works by inhibiting the enzyme aldose reductase, thus decreasing the production of sorbitol and minimizing osmostic stress that is believed to contribute to nerve damage [30]. Another treatment option aimed at decreasing oxidative stress that is present in diabetic neuropathy is the use of antioxidants, such as α-lipoic acid. This has shown limited success in the United States at reducing pain, praresthesias and numbness associated with painful diabetic neuropathy, although no significant improvement in clinical outcomes were evident in two separate clinical trials [35, 36]. Recently, adenosine receptors have gained attention as a

possible target for modulating pain associated with inflammatory and neuropathic pain conditions [81, 173, 174].

Human studies investigating the efficacy of adenosine at alleviating symptoms of pain have shown positive results. One study reports improvement in spontaneous and evoked pain while receiving intravenous (i.v.) infusion of adenosine [87]. Another study reports improvement in tactile pain thresholds [89], and yet another study reports a decrease in the area of mechanical allodynia and evoked pain to mechanical stimuli following intrathecal (i.t.) administration of adenosine [88]. Collectively, these results suggest that adenosine may have beneficial effects in humans for treating pain conditions; however, none of these trials evaluated the effect of adenosine on painful diabetic neuropathy. Furthermore, use of i.v. or i.t. infusion for the delivery of pain medication is not a suitable method of delivery for daily maintenance of pain symptoms for individuals living with painful diabetic neuropathy.

Use of oral medications, as well as i.v. or i.t. delivery of therapeutics, comes with significant risk of systemic adverse effects. Thus, there is a recent impetus to move away from oral medications towards the exploration the use of topical delivery methods for treating painful conditions [45]. In this study, we determined if peripheral delivery of A_1R agonists could activate A_1R-mediated antinociceptive pathways to alleviate signs of mechanical allodynia in a rodent model of painful diabetic neuropathy.

3. Experimental Procedures

Animals

All experiments were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee. Inbred male A/J mice were obtained from Jackson Laboratories at 7 weeks of age, and were induced with diabetes at 8 weeks of age. All mice were housed in the Laboratory Animal Resources building, had *ad libitum* access food and water, and maintained on a 12 hour light/dark cycle. All mice were sacrificed at 16 weeks of age.

Streptozocin induction of diabetes

8 week old male A/J mice were injected with Streptozocin (STZ; Sigma, St. Louis, MO) to induce type 1 diabetes. Mice were fasted for 3 hours before and after injections, for a total fasting time of 6 hours. Injections were spread over 2 days, with the first dose (day 1) at 85 mg/kg and the second dose (day 2) at 65 mg/kg. Solutions were made fresh immediately prior to injection and STZ was dissolved in ice cold 10 mM sodium citrate buffer with 0.9% NaCl at pH 4.5, and then filter sterilized. Control, nondiabetic, mice were injected with sodium citrate buffer only. Animals that did not reach hyperglycemia (blood glucose >230 mg/dL) within one week following initial injections were re-injected with either 85 mg/kg STZ (blood glucose <180 mg/dL) or 65 mg/kg STZ (blood glucose 181-230 mg/dL). Mice that failed to reach hyperglycemia were excluded from the study.

Behavioral analysis

Prior to behavioral testing, mice were acclimated to the behavior facility and equipment for a minimum of 2 days. On test days, mice were placed in the behavior facility and allowed to

acclimate to the environment for at least 30 minutes. Mice were then acclimated on the behavior apparatus for 20 to 30 minutes prior to initiating testing. Mice were placed in individual clear plastic cages on top of a wire mesh grid that allowed access to their hind paws for the duration of the analysis. Mechanical withdrawal thresholds using von Frey monofilaments were measured weekly to track progression of neuropathy phenotype using the up-down method to determine fifty percent withdrawal thresholds [121]. Tests for mechanical sensitivity following intraplantar (i.pl.) injections were performed at 30 min, 1.5 hours, 3 hours and 5 hours following injection.

Drugs and Drug Administration

Adenosine (Ado), AMP and CPA (direct A_1R agonist) were purchased from Sigma; 5'iodotubercidin (ITU; adenosine kinase inhibitor) was purchased from Enzo Life Sciences. AMP, Ado, and CPA were dissolved in 0.9% saline at pH 7.4. AMP was delivered at a concentration of 200 nmol/10 µl [104]; Ado and CPA were delivered at 10 nmol/10 µl [122, 123]; hPAP was dissolved in saline and delivered at a dose of 250 mU/10 µL [100]. ITU was co-administered with Ado or AMP at a concentration of 5 nmol/10 µL [124]. Intraplantar (i.pl) injections (AMP + ITU, Ado + ITU, CPA) were performed using a 31 gauge, ½" insulin syringe (BD Biosciences) between the distal volar footpads of the right hindpaw [175].

Western Blot Analysis

At sacrifice, spinal cord and DRG were flash frozen in liquid nitrogen and stored at -80°C until processing. Tissues were sonicated in cell extraction buffer (Invitrogen, Carlsbad, CA) containing 55.5 µl/mL protease inhibitor cocktail (Sigma, St. Louis, MO), 200 mM Na_3VO_4 and 200 mM NaF for 60 min on ice with gentle vortexing every 10 min for protein extraction. Following centrifugation at 7000 rpm for 10 min at 4°C, the protein concentration of the

supernatant was determined using the Bradford assay (Bio-Rad, Hercules, CA). Samples were boiled with lane marker reducing sample buffer (Thermo Scientific, Waltham, MA) and stored at -20°C until use. Equal amounts of protein were loaded and separated on a 4-15% gradient Tris-glycine gel (Bio-Rad; 30 mA/gel, 45 min, 4°C), and then transferred to a nitrocellulose membrane (400 mA, 1.5 hours, 4°C). Following incubation with primary and secondary antibodies, bands were visualized using Enhanced Chemiluminesence reagent (ECL; Thermo Scientific). Membranes were exposed to x-ray film and analyzed using Image J software (NIH). The primary antibody used was rabbit anti-A_1R (1:500; Thermo Scientific), and the secondary antibody, coupled to horseradish peroxidase, was goat anti-rabbit (Santa Cruz). Bands were visualized with ECL, membranes exposed to x-ray film and analysis completed using Image J software (NIH).

Determination of A_1R mRNA concentration

RNA was extracted from spinal cord, DRG and skin using TRI Reagent (Sigma) and RNeasy Mini Kit (Qiagen, Valencia, CA). Sample concentration and purity were determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Complimentary DNA (cDNA) was synthesized from total RNA using the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using SsoFast Probes Supermix Kit (Bio-Rad). The primers used were as follows:

A_1R: Forward: 5’ – TGTGCCCTTAAATGTACTGG – 3’

Reverse: 5’ – TCTGTGGCCCAATGTTGATAAG – 3’

GAPDH: Forward: 5’ – AGGTCGGTGTGAACGGATTTG – 3’

Reverse: 5’ – TGTAGACCATGAGTTGAGGTCA – 3’

All reactions were performed in triplicate. A_1R mRNA levels (skin) were normalized to GAPDH. Raw PCR data was analyzed using LinRegPCR software (version 2012.3) to determine PCR efficiency values. Threshold cycle values for L27 were subtracted from the samples, and the percentage of fold change from nondiabetic animals was calculated using the pfaffl method.

Statistical analysis

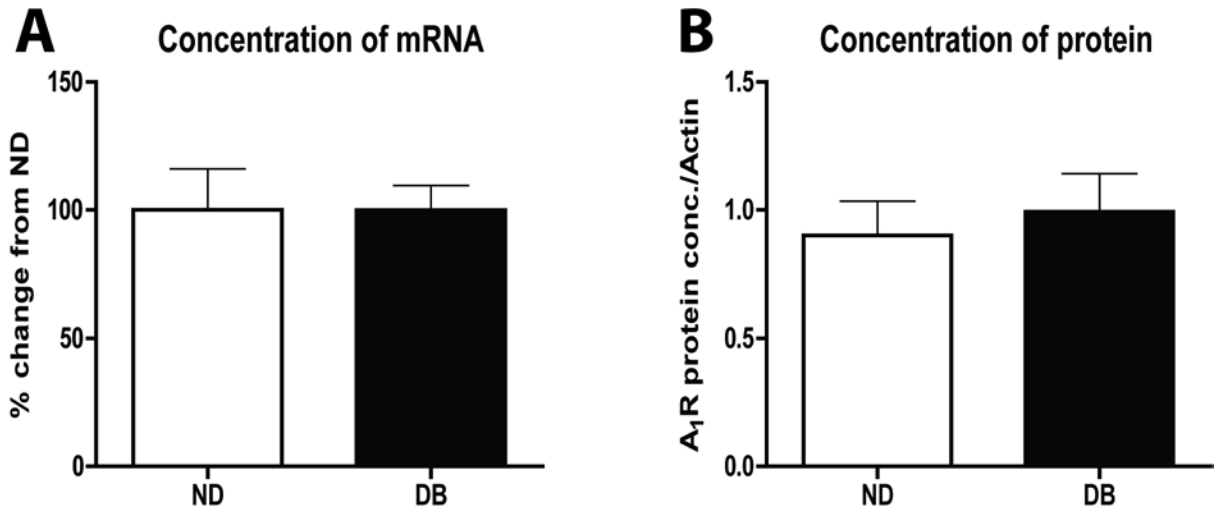
Results were analyzed using the SPSS Statistics 20 software (IBM). Student's t-tests and two-way repeated measures analysis of variance (2-way RM-ANOVA) with Fisher's least significant difference (LSD) post-hoc analyses were performed, as denoted in the manuscript. A *p*-value less than 0.05 was considered significant. All data are presented as mean ± S.E.M.

4. Results & Figures

A_1R is expressed in the hind paw skin of mice

The concentration of A_1R mRNA and protein levels in the skin was evaluated to determine if this important antinociceptive pathway was accessible from the periphery. Analysis of mRNA expression levels in the skin revealed that A_1R is detectable in the hind paw; however, there were no differences in relative expression of A_1R mRNA levels between nondiabetic and diabetic mice (Fig. 22A). Protein expression levels were also evaluated, and no significant differences were detected between nondiabetic and diabetic mice (Fig. 22B).

Figure 22. A_1R is expressed in the hind paw of mice. A) Expression of A_1R mRNA was detected in the hind paw skin of nondiabetic (ND) and diabetic (DB) mice, but there was no difference in the relative expression levels of A_1R mRNA between the two groups ($p > 0.05$ vs. ND; t-test). B) Protein expression of A_1R not significantly different between ND and DB mice ($p > 0.05$ vs. ND; t-test). n = 7 mice per group.


A
Concentration of mRNA
% change from ND
150
100
50
0
ND
DB
B
Concentration of protein
A1R protein conc./Actin
1.5
1.0
0.5
0.0
ND
DB

Peripheral delivery of an A_1R agonist reduces mechanical hypersensitivity.

Because A_1R is expressed in the skin, we hypothesized that peripheral delivery of A_1R agonists may be able to activate this antinociceptive pathway following a peripheral route of administration. Protein expression of NT5E, but not PAP, has been detected in the hind paw skin of mice [104]. Thus, i.pl. AMP + ITU was tested to determine if endogenous enzymatic hydrolysis of AMP was sufficient to activate peripheral A_1R. Unfortunately, i.pl. administration of AMP + ITU was not effective at alleviating mechanical allodynia in diabetic mice (Fig. 23).

To bypass enzymatic hydrolysis, i.pl. Ado + ITU was tested to determine if direct activation of A_1R could alleviated mechanical allodynia in diabetic mice. This, too, failed to alleviate mechanical allodynia in diabetic mice; rather, diabetic mice remained consistently allodynic throughout the duration of the study (Fig. 24). Diabetic mice treated with i.pl. Ado + ITU had significantly reduced withdrawal thresholds compared to diabetic vehicle treated mice at 30 min and 1.5 hours following treatment. Interestingly, nondiabetic mice treated with i.pl. Ado + ITU were not significantly different from diabetic mice treated i.pl. Ado + ITU at 30 min and 1.5 hours, but were significantly different at 3 hours post-treatment and trending towards significantly different at 5 hours ($p = 0.065$) post-treatment.

Figure 23. Peripheral delivery of AMP + ITU does not alleviate mechanical allodynia in diabetic mice. Diabetic (DB) mice did not show any recovery in their mechanical withdrawal threshold following administration of i.pl. AMP + ITU (AMP, 200 nmol/10 µl; ITU, 5 nmol/10 µl) compared to DB vehicle treated mice ($p > 0.05$ vs. DB Veh; 2-way RM-ANOVA with LSD post-hoc analysis). n = 5-7 mice per group.

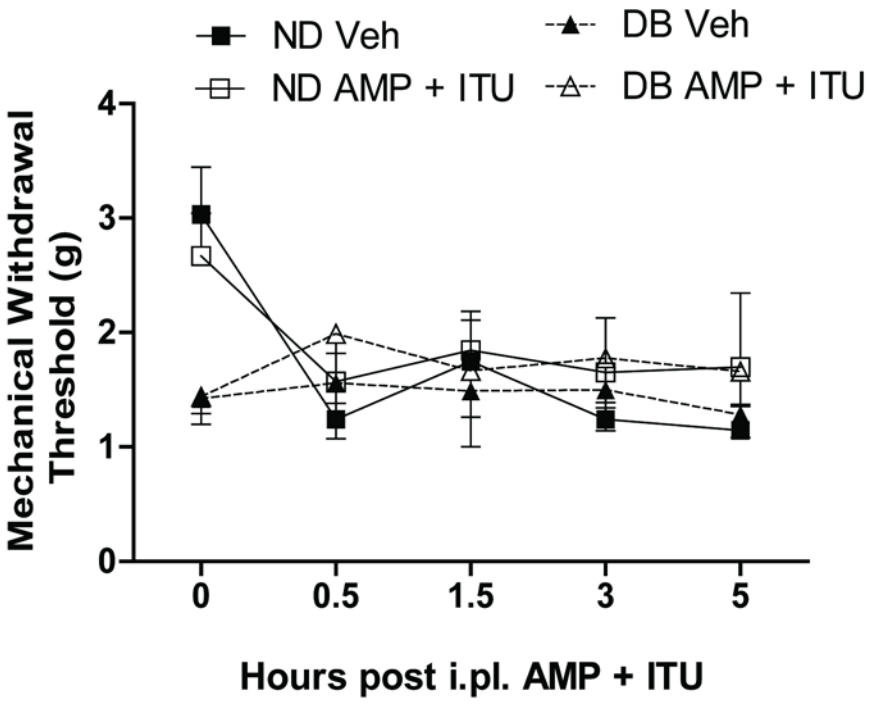
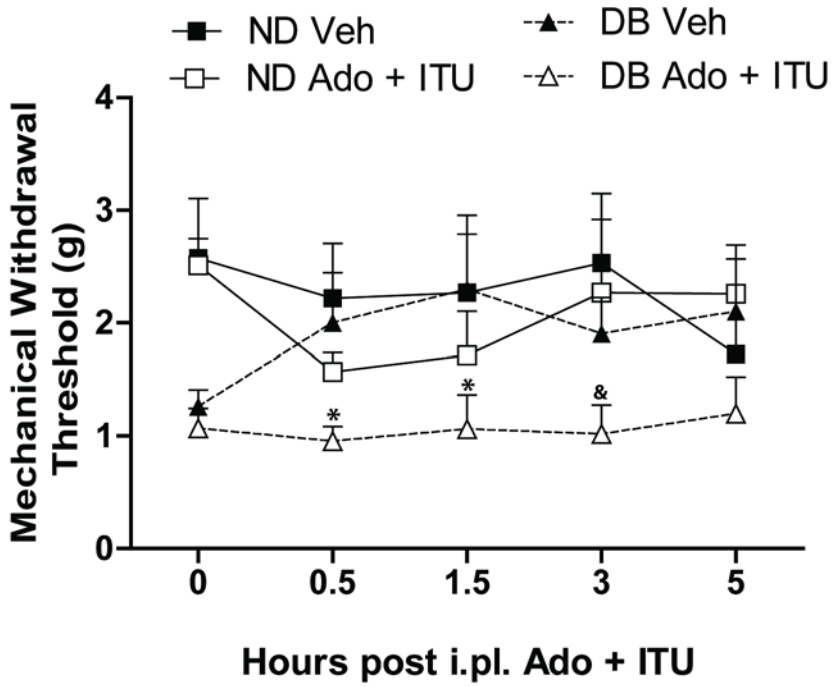

ND Veh
ND AMP + ITU
DB Veh
DB AMP + ITU
Mechanical Withdrawal Threshold (g)
0
1
2
3
4
0
0.5
1.5
3
5
Hours post i.pl. AMP + ITU

Figure 24. Administration of i.pl. Ado + ITU did not reverse mechanical allodynia in diabetic mice. Diabetic (DB) mice treated with i.pl Ado + ITU (Ado, 10 nmol/10 µl; ITU, 5 nmol/10 µl) remained consistently allodynic throughout the duration of the experiment, and had significantly reduced withdrawal thresholds compared to DB vehicle treated mice at 30 min and 1.5 hours post-treatment (* $p < 0.05$ vs. DB Veh; 2-way RM-ANOVA with LSD post-hoc analysis). Nondiabetic (ND) mice treated with i.pl. Ado + ITU were not significantly different from DB i.pl. Ado + ITU (DB Ado) treated mice at 30 min and 1.5 hours following treatment, but were significantly different at 3 hours ($^{\&}$ $p < 0.05$ vs. DB Ado; 2-way RM- ANOVA with LSD post-hoc analysis). n = 5 – 8 mice per group.


ND Veh
ND Ado + ITU
DB Veh
DB Ado + ITU
Mechanical Withdrawal Threshold (g)
0
1
2
3
4
0
0.5
1.5
3
5
*
*
&
Hours post i.pl. Ado + ITU

Finally, the direct A_1R agonist, CPA, was given to determine if specific activation of A_1R had any effect on mechanical allodynia. Surprisingly, i.pl. CPA was able to transiently reverse mechanical sensitivity in diabetic mice for up to 3 hours compared to baseline and diabetic vehicle treated mice (Fig. 25). Furthermore, nondiabetic mice that received i.pl. CPA displayed mechanical withdrawal thresholds that were significantly elevated compared to nondiabetic vehicle treated mice for up to 3 hours, similar to diabetic CPA-treated mice.

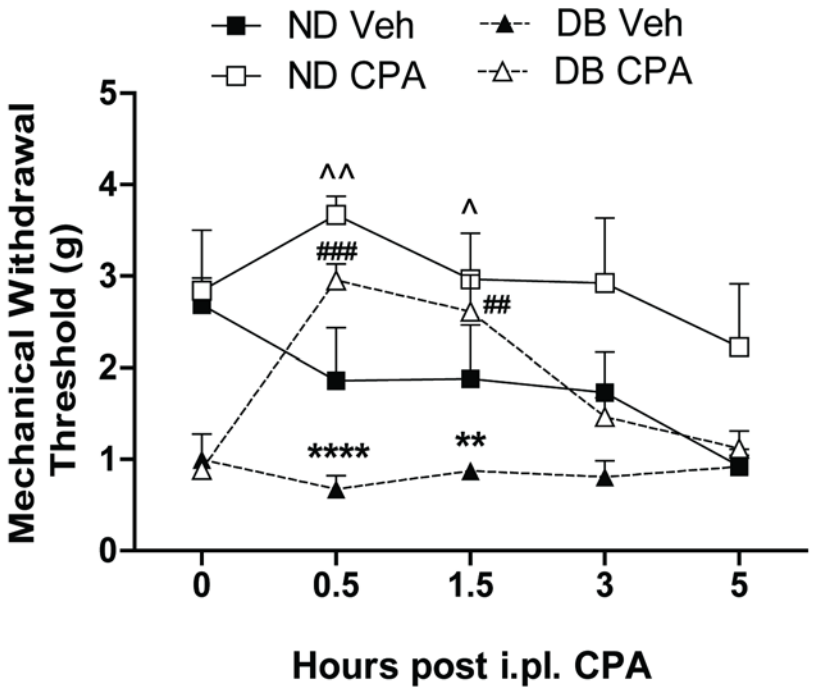
5. Discussion

In this study, the efficacy of peripheral delivery of A_1R agonists was evaluated. Results show that direct peripheral activation of A_1R by the selective agonist, CPA, provided relief of mechanical allodynia in diabetic mice for up to 3 hours. Furthermore, nondiabetic mice that received the active compound had significantly elevated mechanical withdrawal thresholds over nondiabetic mice that received vehicle only. These results show that peripheral activation of A_1R-mediated antinociceptive pathways may provide a novel mechanism for treatment of painful diabetic neuropathy that bypasses traditional systemic (oral) delivery routes.

Adenosine in the periphery is pro-nociceptive

Previous studies have shown that direct administration of adenosine to the hind paw of rats results in cutaneous hyperalgesia and decreased paw withdrawal thresholds, a result likely mediated through activation of $A_{2A}R$ and increased cAMP production [176]. In this study,

Figure 25. Peripheral delivery of the A_1R agonist, CPA, significantly reverses mechanical allodynia in diabetic mice. Intraplantar administration of CPA (10 nmol/10 µl) significantly reversed mechanical allodynia in diabetic (DB) mice compared to baseline (## $p < 0.01$ vs. DB CPA; 2-way RM-ANOVA with LSD post-hoc analysis) and compared to DB vehicle treated mice (** $p < 0.01$ vs. DB Veh; 2-way RM-ANOVA with LSD post-hoc analysis). Additionally, nondiabetic mice (ND) that received i.pl. CPA had significantly increased withdrawal thresholds compared to ND vehicle treated mice for up to 3 hours (^^ $p < 0.01$ vs. ND Veh; 2-way RM-ANOVA with LSD post-hoc analysis). n = 4 – 5 mice per group.


ND Veh
ND CPA
DB Veh
DB CPA
Mechanical Withdrawal Threshold (g)
0
1
2
3
4
5
^^
^
###
##
****
**
0
0.5
1.5
3
5
Hours post i.pl. CPA

diabetic mice that received peripheral (i.pl.) delivery of AMP + ITU did not show any recovery in mechanical withdrawal thresholds, suggesting that indirect activation of A_1R by NT5E-mediated hydrolysis of AMP was insufficient to activate this antinociceptive pathway. Additionally, all nondiabetic mice, including both AMP-treated and vehicle-treated mice, displayed significantly reduced withdrawal thresholds compared to baseline. This suggests that the injection itself may have caused significant sensitivity as evidenced by decreased withdrawal thresholds in nondiabetic vehicle-treated mice, and further supports the conclusion that indirect activation of A_1R is ineffective at alleviating mechanical sensitivity.

Further evidence that administration of adenosine in the periphery is pro-nociceptive is seen in nondiabetic mice that received i.pl. Ado + ITU. These mice display a slight decrease in their mechanical withdrawal thresholds that were not significantly different from diabetic mice who also received i.pl. Ado + ITU. Diabetic mice that received i.pl. Ado + ITU remained consistently allodynic throughout the duration of the study, whereas diabetic mice that received only the vehicle showed improvement in their withdrawal thresholds, although this was not significantly different from baseline. Additionally, nondiabetic mice that received only the vehicle had no change in their withdrawal thresholds, supporting the notion peripheral administration of adenosine increased mechanical sensitivity in nondiabetic mice. Futures studies should investigate the contribution of other peripherally located adenosine receptors, specifically $A_{2A}R$, to the development of mechanical sensitivity following peripheral delivery of adenosine.

Peripheral delivery of a direct A_1R agonist alleviated mechanical allodynia in diabetic mice

Previous studies have shown that peripheral delivery of the A_1R agonist, CPA, can inhibit the peripheral pro-nociceptive effects of adenosine, $A_{2A}R$ activation and PGE_2-mediated hyperalgesia is the rat hind paw [176, 177]. The results presented here directly support this theory, as evidenced by a reversal of mechanical withdrawal thresholds in diabetic mice treated with i.pl. CPA. Additionally, nondiabetic mice that received i.pl. CPA displayed mechanical withdrawal thresholds that were significantly elevated over nondiabetic mice that received only the vehicle. It should be noted that in this study, nondiabetic vehicle-treated mice developed decreasing withdrawal thresholds throughout the experiment which could be due to several mechanisms, including learned behavior in the absence of an analgesic on board and in the face of repeated stimulation, inflammatory pain as a result of the injection itself, or a behavioral anomaly in this specific experimental group.

Recently, there has been an impetus to move away from oral medications for pain management and a push towards topical administration of analgesics to mitigate systemic adverse effects [45, 178, 179], which is one of the major limitations of current treatment options [17, 114, 117]. There have been a number of human studies demonstrating efficacy in using adenosine as a potential analgesic [88, 173, 180]. Many of these studies involve either i.v. or i.t. administration of adenosine, but this, too, has limitations including headache and transient low back pain at the sight of injection [87, 88, 173, 180]. These studies provide further evidence that peripheral delivery of a direct A_1R agonist can provide analgesic relief of pain-like symptoms in a mouse model of painful diabetic neuropathy.

Conclusions

The experiments performed in this chapter were done in an attempt to translate the encouraging outcomes seen following i.t. administration of A_1R agonists into a potential therapy for individuals suffering from painful diabetic neuropathy. Indirect hydrolysis of the substrate for NT5E, AMP, was ineffective at activating A_1R-mediated antinociceptive pathways, nor was peripheral delivery of adenosine. In fact, peripheral delivery of adenosine revealed outcomes that suggest adenosine in the periphery may have pro-nociceptive actions, a result opposite of the goals of this study. Surprisingly, peripheral delivery of the specific A_1R agonist, CPA, effectively reversed mechanical allodynia in diabetic mice. This suggests that directly targeting A_1R with agonists specific for this receptor may provide a novel mechanism to alleviate mechanical allodynia, warranting further investigation into the efficacy of topical analgesics that activate A_1R for treating painful diabetic neuropathy and other neuropathic pain conditions.

CHAPTER 5

General Discussion and Conclusions

Purinergic signaling has been studied for several decades, and significant advances have been made towards the understanding of adenosine and adenosine receptors in normal physiological and pathophysiological processes, and the potential for these signaling pathways to be utilized as targets for treatment options. Activation of A_1R has been explored as a potential therapeutic target due to its antinociceptive effects, and it is this feature that was exploited in this dissertation to determine the role A_1R in modulating pain sensation in a rodent model of painful diabetic neuropathy. Through a series of *in vitro* and *in vivo* experiments, results contained herein show that diabetes significantly affects the ability of ectonucleotidases to generate extracellular adenosine, but that this important antinociceptive pathway is still functional and can be activated through central and peripheral delivery methods to alleviate signs of mechanical allodynia in diabetic mice.

Diabetes affects generation of extracellular adenosine

Extracellular adenosine primarily arises from two sources: release from intracellular stores through equilibrative nucleotide transporters in the cell membrane, or through extracellular hydrolysis of adenine nucleotides by ectonucleotidases. Extensive studies have identified PAP and NT5E as the primary enzymes in the spinal cord and DRG responsible for hydrolyzing AMP, and these two enzymes are primarily located on small- and medium-diameter, putative nociceptive neurons, placing them in prime location to modulate nociceptive neurotransmission [100, 103-105]. Studies using A_1R knockout mice ($A_1R^{-/-}$) provided some of the first *in vivo* evidence that A_1R has a physiological role in modulating nociception [79, 86]. These studies also revealed decreased inhibition of excitatory glutamatergic signaling, evidenced by a lack of

inhibition of field excitatory postsynaptic potentials in the hippocampus of $A_1R^{-/-}$ mice. Interestingly, mice heterozygous for A_1R ($A_1R^{+/-}$) showed loss of inhibition that was proportional to the number of A_1R receptors expressed. Furthermore, administration of i.t. hPAP and recombinant NT5E were ineffective at alleviating mechanical allodynia and thermal hyperalgesia in $A_1R^{-/-}$ mice following induction of inflammatory or neuropathic pain models, confirming that the antinociceptive effects of adenosine generated through hydrolysis of AMP by ectonucleotidases is mediated through activation of A_1R [100, 124]. Subsequent studies have confirmed that adenosine modulates nociceptive neurotransmission in the substantia gelatinosa through control of glutamate release from spinal interneurons, thus regulating excitatory synaptic transmission [112, 118, 120].

At eight weeks post-STZ induction of diabetes, male A/J diabetic mice display significant mechanical allodynia as evidenced by significantly decreased mechanical withdrawal thresholds compared to nondiabetic mice, indicative of a pain-like phenotype. At this same time point, we show for the first time that diabetes significantly affects the endogenous hydrolytic capacity of ectonucleotidases in the DRG at physiological pH (7.4) and acidic pH (5.6) to generate adenosine from AMP, suggesting that altered adenosine production and loss of activation of A_1R may contribute to the development of painful diabetic neuropathy in this model system. At this same time point, A_1R protein expression levels remain unchanged in the DRG of diabetic mice compared to nondiabetic mice. However, in the spinal cord, A_1R protein expression levels were trending towards a significant decrease in diabetic mice compared to nondiabetic controls, yet there was no change in the hydrolytic capacity of ectonucleotidases in the spinal cord. Thus, in

the DRG, A_1R expression levels likely remain similar to control levels due to decreased bioavailability of adenosine locally. Given that ectonucleotidase activity in the spinal cord of diabetic mice is comparable to control levels, an overall decrease in adenosine receptor expression could influence nociceptive tone in the central nervous system through decreased activation of A_1R as a result of down-regulation of the receptor. Collectively, these data suggest that decreased adenosine production in the peripheral nervous system (DRG) coupled with fewer receptors available for activation in the central nervous system (spinal cord) could have a major impact on pain transmission, manifested as mechanical allodynia in our model of painful diabetic neuropathy.

To further investigate the role of ectonucleotidases and their contribution to the development of painful diabetic neuropathy, measuring *in vivo* levels of adenosine would provide direct evidence regarding fluctuations in endogenous production of adenosine in the setting of diabetes. Street et al. were able to measure in real-time the production of adenosine following application of 100 μM AMP to the dorsal horn of spinal cord slices from mice using fast-scan voltammetry [105]. This method would add further evidence that ectonucleotidase activity is altered as a consequence of diabetes, and could likely be adapted to measure adenosine production in the DRG. Alternatively, high performance liquid chromatography or tandem mass spectroscopy could provide significant insight into the levels of adenosine in the spinal cord and DRG in diabetic and nondiabetic mice [181-183]. This could also be used to measure adenosine production following treatment of diabetic mice with i.t. hPAP and i.t. AMP + ITU.

The other side of the adenosine-A_1R-antinociception spectrum is the contribution of P2 receptors (P2X and P2Y) to pain sensation and transmission, which are activated primarily by ATP and ADP [68, 184, 185]. ATP-mediated signaling is well documented in the transduction of painful stimuli, and numerous studies have identified $P2X_3$ as one of the main receptors involved in this process [185-187]. Interestingly, mRNA levels of $P2X_3$ in the DRG are elevated in STZ-induced diabetic rats, and diabetic rats display signs of mechanical allodynia [187], and inhibition of $P2X_3$ has been shown to alleviate mechanical allodynia in diabetic rats [188]. Not surprisingly, PAP and NT5E are also located on this same neuronal population [100, 103, 104], strengthening the argument that the role of these enzymes is to mitigate pro-nociceptive signaling induced by ATP.

More recently, $P2X_4$ has been under investigation for its potential role in the development of mechanical allodynia. Tsuda et al. showed that $P2X_4$ receptor expression is upregulated in activated microglia following nerve injury rats [71]. Inhibitors of both $P2X_3$ and $P2X_4$ were effective at reversing mechanical allodynia in these models, suggesting another potential target for the treatment of painful diabetic neuropathy. Diabetes is associated with increased inflammation, and ATP-mediated signaling is increased under inflammatory conditions [127, 189]. Taken together, it is plausible to suggest that in diabetes the scale is tipped towards a pro-nociceptive state. Up-regulation of P2X receptors, coupled with decreased hydrolysis of AMP and generation of adenosine, results in decreased activation of A_1R and the downstream antinociceptive pathways, thus allowing for signaling propagation through pro-nociceptive P2X receptors.

Additionally, P2Y receptor-mediated signaling is also involved in pain transmission. These receptors are activated primarily by ATP or ADP, although some can also respond to UTP or UDP [72, 184]. $P2Y_1$ receptors were shown to be expressed in a majority of small-diameter neurons, specifically neurons that express IB4 and $P2X_3$ [190], and $P2Y_1$ is necessary in the development of CFA-induced inflammatory pain [191]. Additionally, $P2Y_{12,}$ $P2Y_{13}$ and $P2Y_{14}$ are expressed on small-diameter, putative nociceptive neurons, and activation of these receptors via ADP is involved in mitigating CFA-induced inflammatory pain [191], further adding to the complexity of nociceptive neurotransmission and inhibition. Much less is known about the involvement of P2Y receptors in pain transmission and inhibition compared to P2X receptors, and even less is known about the effect of diabetes on P2Y receptor expression and function. Evaluation of P2Y receptor expression and function in the context of diabetes would add a substantial body of knowledge to the field of purinergic signaling in general, particularly in the context of painful diabetic neuropathy, and would aid in our understanding of the role of purinergic signaling in the development and inhibition of painful diabetic neuropathy.

Central delivery of A_1R agonists can alleviate mechanical allodynia in painful diabetic neuropathy

Of the four recognized adenosine receptors, A_1R is attributed with having antinociceptive benefits. Indeed, this has been documented in numerous studies exploring the role of A_1R activation in mitigating pain transmission in rodent models [131-133, 192-194]. The data contained herein adds another piece to the puzzle by showing that central activation of A_1R is effective at alleviating mechanical allodynia in a rodent model of painful diabetic neuropathy.

Most studies investigating the role of A_1R-mediated antinociception use artificial methods to induce nociception using animal pain models, such as induction of inflammatory pain by CFA, carrageenan or formalin, or surgical methods, such as sciatic nerve ligation or chronic constriction injury, to induce neuropathic pain. Our model consistently results in increased mechanical sensitivity as the course of this chronic, systemic disease progresses. Furthermore, most studies show an effect of A_1R activation on alleviating thermal hyperalgesia, and far fewer studies explore the contribution of A_1R activation and modulation of mechanical sensation. The work presented in this dissertation clearly demonstrates that A_1R activation does have an effect on mechanical sensation, and that central activation of this receptor is effective at alleviating mechanical sensitivity in diabetic mice.

To truly confirm that the antiallodynic effects seen in response to i.t. administration of AMP + ITU, Ado + ITU and CPA are mediated through A_1R, future studies should incorporate the use of $A_1R^{-/-}$ mice back-bred onto an A/J background. $A_1R^{-/-}$ mice are commercially available from Jackson Laboratories on a C57Bl/6 background. Use of conditional (doxycycline-inducible deletion) or selective (peripheral nervous system specific Cre/lox activated elimination) A_1R knockout mice could provide additional insight into the contribution of A_1R-mediated antinociception in the central and/or peripheral nervous system. Additionally, future *in vitro* and *in vivo* experiments should include the use of antagonists to A_1R, such as the modified xanthine derivative, DPCPX, to add further evidence that the behavioral outcomes presented in this body of work are indeed mediated through activation of A_1R.

A small number of studies have been conducted in human patients to evaluate the efficacy of i.t. adenosine on alleviating pain, and none have specifically looked at the effect of adenosine on treating signs and symptoms associated with painful diabetic neuropathy [195]. Belfrage et al. evaluated the efficacy of i.t. adenosine and found that spontaneous and evoked pain were reduced in a small cohort of patients, with transient low back pain the only reported side effect [87]. As a follow up, Eisenach et al. evaluated the efficacy of i.t. vs. i.v. delivery of adenosine and found that i.v. administration of adenosine effectively reduced the area of allodynia and evoked pain in the area of allodynia [88]. A clinical trial evaluating the efficacy of a novel $A_{2A}R$ agonist for the treatment of diabetic neuropathic pain was well tolerated by showed no significant improvement in pain symptoms [173]. The work presented here shows that activation of A_1R is effective in treating pain-like symptoms associated with diabetic neuropathy, and warrants inclusion of patients with painful diabetic neuropathy in clinical trials evaluating the efficacy of adenosine as a treatment modality for neuropathic pain.

Decreased cAMP production and increased Akt activation contribute to the antinociceptive effects of A_1R activation following central delivery of A_1R agonists

All adenosine receptors are GPCRs and activation causes dissociation of the associated heterotrimeric G-protein, which further subdivides into an α-subunit and a βγ-subunit. Each of these subunits can then independently activate many different downstream signaling cascades, resulting in major amplification of cellular signals. Classically, A_1R activation is linked to inhibition of andenylyl cyclase through activation of $G\alpha_i$, leading to decreased production of cAMP levels [138]. Interestingly, following i.t. delivery of Ado + ITU, cAMP levels in the

spinal cord were significantly decreased compared to baseline levels, and had still not recovered at 5 hours following treatment. This correlates with the observed behavioral outcomes in which diabetic mice that received i.t. Ado + ITU displayed mechanical withdrawal thresholds that were significantly elevated over diabetic vehicle treated mice, and that were not significantly different from nondiabetic mice, an effect that last over 5 hours. Several substrates and genes are regulated downstream of cAMP, and future studies should investigate the contribution (or inhibition) of these to A_1R-mediated antinociception in the context of painful diabetic neuropathy.

High levels of A_1R protein are seen in adipose tissue, and activation of the receptor reduces the breakdown of triglycerides into free fatty acids (FFAs), an act that is mediated through inhibition of cAMP production and therefore PKA activation, which in turn inhibits the actions of hormone-sensitive lipase and adipose triglyceride lipase [196, 197]. Type 2 diabetes is largely associated with peripheral insulin resistance and obesity [2-5, 28], and individuals with diabetes are more likely to have dyslipidemia than the general population [198]. Dyslipidemia is defined as increased levels of plasma triglycerides, low levels of circulating HDL, and increased levels of LDL, and is associated with the development of cardiovascular disease [199]. Recently, Guilford et al. has shown that diabetic mice fed a high-fat diet results in dyslipidemia and phenotypic switching from an insensate neuropathy to a painful neuropathy, suggesting that circulating levels of FFAs may influence the development of diabetic neuropathy [200]. Combining this observation with the work presented in this document, it would be interesting to explore the effect of adenosine and A_1R activation in a high-fat model of type 2 diabetes, and to

determine if activation of A_1R could also reverse signs of mechanical allodynia in this model of painful diabetic neuropathy.

Currently, a majority of the literature reports activation of Akt as an intracellular mediator of nociceptive neurotransmission. However, we show that Akt is robustly activated following i.t. administration of both Ado + ITU and CPA that correlates with improved mechanical withdrawal thresholds in diabetic mice, and this activation is elevated in diabetic mice compared to nondiabetic mice. This suggests a role of Akt activation in mitigating pain transmission, rather than propagating pain transmission. The A_1R can exist as part of a receptor complex, and has been shown form heteromultimers with μ- and δ-opioid receptors [52, 177, 201]. Activation of Akt is seen in the periphery following administration of μ-opioid agonists, resulting in inhibition of inflammatory hyperalgesia [164, 165]. Clearly, further understanding of the role of Akt signaling in mediating the antinociceptive outcomes observed following central activation of A_1R is needed.

Calcium channel expression and signaling is dysregulated in painful diabetic neuropathy

Calcium channel signaling is involved in many physiological processes, and is intimately involved in neuronal function, including modulation of neuronal excitability and neurotransmitter release [150, 202]. Furthermore, calcium channel dysregulation is strongly associated with neuronal disease, particularly in the case of diabetic neuropathy [150]. Kostyuk et al. report increased $[Ca^{2+}]_i$ in neurons from diabetic mice and rats, suggesting that diabetes has a general effect on the ability of neurons to regulate intracellular calcium stores in the setting of diabetes. At 8 weeks post STZ-induction of diabetes, we showed increased calcium flux through

neurons following stimulation with the TRPV1 agonist, capsaicin, supporting the notion that diabetes affects calcium signaling in neurons from diabetic animals.

It should be noted that this analysis included all cells that responded to capsaicin, not just small-diameter neurons, although this subset of neurons is primarily the subset that expresses TRPV1. Initial experiments attempted to label $IB4^{+}$ neurons, however, the presence of the antibody interfered with calcium signaling and data acquisition; these experiments were not included in data analysis. Back-labeling of neurons with DiI would allow identification of neurons that project to the hind paws of diabetic mice, thus allowing evaluation of calcium flux through this specific population. However, this presents its own unique set of challenges in the face of diabetic neuropathy given the neurodegenerative nature of diabetic neuropathy and the 'dying-back' phenomenon. Nonetheless, it would allow for determination of changes in calcium signaling in surviving neurons from diabetic mice. Additionally, these experiments were performed at a terminal time point, at which time diabetic mice displayed significant allodynia. Determination of changes in calcium signaling at earlier time points would shed light on the time course of diabetes-induced changes in calcium homeostasis. The use of specific agonists and antagonists would add further insight into the role of specific calcium channels and receptor-specific modulation of calcium signaling.

Activation of A_1R is known to inhibit P-, Q- and N-type calcium channels [81]. Dysregulation of N-type ($Ca_v2.2$) channels has been reported in diabetic rats, and this dysregulation was associated with impaired G-protein modulation of calcium channel signaling [167]. Here, were show that diabetes affects generation of adenosine (through inhibition of

ectonucleotidase-mediated hydrolysis of AMP), which in turn results in decreased activation of A_1R, leading to decreased G-protein modulation of calcium channels and dyregulation of calcium homeostasis. Indeed, we show that incubation of primary DRG cultures with adenosine returns the increased calcium flux in neurons from diabetic mice to control levels, supporting the notion that calcium channels are regulated by G-proteins, specifically the βγ-subunit [203]. Repeating these experiments with the A_1R specific agonist, CPA, would further support the claim that decreased A_1R activation, as a result of decreased adenosine production, leads to altered calcium homeostasis subsequent to decreased G-protein inhibition of calcium channel signaling. Additionally, use of βγ-subunit specific inhibitors would add tremendous support to this theory.

Finally, several studies report upregulation of the $\alpha_2\delta$-subunit of calcium channels following nerve injury [151, 169, 170, 204]. Interestingly, this is the molecular target for gabapentin and pregabalin [205]. We evaluated protein levels of the α_2-subunit (the α_2-subunit and the δ-subunit dissociate under reducing conditions used for Western blot analysis) and did not see any significant changes in diabetic mice. However, these studies were conducted at a terminal time point so it is possible that temporal regulation affects the window of detection for upreglation of the $\alpha_2\delta$-subunit of calcium channels.

Peripheral delivery of specific A_1R agonist reveals a novel mechanism for the treatment of painful diabetic neuropathy

First-line treatment options for neuropathic pain, as recommended by the Neuropathic Pain Special Interest Group of the IASP, include tricyclic antidepressants, serotonin-norepinephrine reuptake inhibitors, calcium channel modulators (ex: gabapentin and pregabalin) and topical lidocaine [33]. Current treatment options for painful diabetic neuropathy include the use of all of these agents, with gabapentin, pregabalin and duloxetine topping the list as the most effective agents [117]. However, the efficacy of these medications varies between individuals, and many carry significant risk of systemic adverse effects [17, 34, 206]. Ulugol et al. report that the anti-allodynic effect of amitriptyline in diabetic rats involves adenosinergic signaling [207], providing some of the only evidence to date that adenosine is effective at alleviating mechanical allodynia associated with diabetic neuropathy.

Data presented in this dissertation support the theory that peripheral administration of adenosine is pro-nociceptive. Diabetic mice that received i.pl. Ado + ITU remained allodynic throughout the duration of the experiment, and nondiabetic mice treated with i.pl. Ado + ITU hewed a decreased in withdrawal thresholds that was not significantly different from diabetic Ado + ITU treated mice. Surprisingly, we found that peripheral activation of A_1R with the specific A_1R agonist, CPA, effectively alleviated mechanical allodynia in diabetic mice. This experiment should be repeated to increase the number of mice included in this study, as the current study was the last behavioral experiment conducted and fewer than anticipated mice survived to the time point at which the pharmacological interventions were performed.

The results of this study are extremely encouraging when considering translation of this body of work into potential treatment options for the management of painful diabetic neuropathy. The behavioral outcomes seen following i.pl. administration of CPA warrant further investigation into peripheral activation of A_1R-mediated antinociceptive pathways for the treatment of painful diabetic neuropathy. Topical creams, such as capsaicin and lidocaine, have shown success for the treatment of other neuropathic pain syndromes, including painful diabetic neuropathy, PHN, and HIV-induced neuropathy [33, 45]. The data presented here are currently one of the only *in vivo* studies to show that A_1R activation in the periphery is an effective target for the treatment of painful diabetic neuropathy. When translating this body of work to humans, knowing that this antinociceptive pathway is accessible from periphery allows future development of treatment regimens which take advantage of a peripheral route of delivery rather than a systemic route (oral or i.v.), thus minimizing the potential for the development of systemic adverse effects. To avoid injection-induced tissue injury and inflammation, development of topical creams that allow for diffusion of CPA or other A_1R-specific agonists into the skin would have major therapeutic treatment potential not only for painful diabetic neuropathy, but presumably many types of peripheral pain syndromes.

Overall Conclusions

The work presented in this dissertation contributes significant understanding into the role of adenosinergic signaling in the context of painful diabetic neuropathy. Adenosine is an important neuromodulator involved in many processes related to nervous system health and disease. Adenosine receptors are ubiquitously expressed throughout the body, with certain

receptors more prominent in specific tissues. Specifically, A_1R is highly concentrated in the DRG and spinal cord, and activation of this receptor is involved in counteracting pro-nociceptive puringergic signaling. The work present herein shows that diabetes significantly affects endogenous adenosine production, thus releasing nociceptive neurons from tonic inhibitory regulation, in addition to altered expression of A_1R in the central nervous system of diabetic mice. Pathway function is conserved, however, because central and peripheral activation of A_1R results in significant improvement of mechanical withdrawal thresholds in diabetic mice. Furthermore inhibition of cAMP production and activation of Akt were identified as potential downstream messengers involved in mediating these antinociceptive outcomes. Finally, improved mechanical withdrawal thresholds following peripheral delivery of an A_1R-specific agonist shows that activation of this pathway has practical applications for translation of this work into a therapy for individuals suffering from painful diabetic neuropathy.

CHAPTER 6

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